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SECTION MEETINGS

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Prerequisites of Successful Heparinization to Prevent Gangrene After Frostbite.* (17790)

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Lange and Boyd(1) demonstrated by use of the fluorescein method, that gangrene after frostbite is due to a tremendous increase in capillary permeability which leads to loss of plasma from the blood stream into the tissue so that the red cells silt up the capillaries due to loss of suspending fluid. This red cell sludge solidifies and is finally converted into hyaline thrombi. These masses of agglutinated red cells obstruct the blood stream locally and lead to the gangrene of the dependent tissue. Lange and Boyd(1) first described that heparinization shortly after exposure and to the extent that coagulation time (Lee-White method) is never shorter than 22 minutes, prevented occurrence of gangrene in 82% of frostbitten rabbit legs. Under these conditions the red cell sludge does not become compact nor adherent to the wall but redissolves. Laufmann *et al.*(2) confirmed our findings in an entirely different set-up. He stated that red cell sludge is not transformed into masses adhering to the vascular wall when sufficient amounts of heparin are given. The necessity for an uninterrupted and frequently checked maintenance of prolonged coagulation time to prevent gangrene has been stressed by us repeatedly(3,4).

Quintanilla *et al.*(6) were not able to prevent gangrene after frostbite in rabbits, but they continued their treatment for 36 hours only, without check on coagulation time and

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* Carried out under grants from the John and Mary R. Markle Foundation and later under contracts among the Office of Scientific Research and Development of the National Research Council and the Office of the Surgeon General, U. S. Army, and the New York Medical College.

1. Lange, K., and Boyd, L. J., *Surg., Gynec., and Obst.*, 1945, v80, 346.

2. Laufmann, H., Martin, W. B., and Tanturi, C., *Science*, 1948, v108, 283.

3. Lange, K., Weiner, D., and Boyd, L. J., *Am. Heart J.*, 1948, v35, 238.

4. Lange, K., Weiner, D., and Boyd, L. J., *New England J. Med.*, 1947, v237, 383.

6. Quintanilla, R., Krusen, F. H., and Essex, H. E., *Am. J. Physiol.*, 1947, v149, 149.

with apparently insufficient doses. They tried to prevent the sludge formation by heparin injections. Sludging, however, is not prevented by heparin at all as shown by Laufmann(2).

Lempke and Shumacker(7) showed that in mice whose tails were exposed to frostbite, a dose of heparin every 3 hours for 3 days after exposure completely prevented gangrene in 39% of the animals. Another 39% showed minimal lesions. The control group showed only 4.8% recovery. If the interval between the heparin injections, however, was extended to 4 hours, the loss of tissue was almost 3 times as great as in the group receiving heparin at shorter intervals. No animals survived without gangrene. After 3 hours, the coagulation time of most of the animals was still markedly elevated, while after 4 hours, the majority of them had returned close to normal. This illustrates that good results can be obtained only if the coagulation time is not permitted to return close to normal.

Fuhrman and Crismon(8) have attempted to prevent gangrene subsequent to frostbite in 3 rabbits by injecting 10 mg/kg of heparin for 3 days at 24, 12 or 8 hour intervals respectively. This dosage is completely insufficient as shown by Shumacker(9) as well as by us. The results obtained are, therefore, without significance.

Lewis(10) demonstrated that by giving heparin initially and following up with dicumarol, he was able to save 60% of the tissue that sloughed off in the controls. No data about the dosage are available. It is difficult to say whether it was sufficient to produce a continuous and ample prolongation of coagulation or prothrombin time respectively.

Most recently, Pichotka and Lewis(11) concluded that the use of heparin in frostbite is of no value. In a previous personal communication, Pichotka had told us that he has been unable to produce a continuous prolongation of coagulation time by the dosage we used. We are not able to explain these differences. Different strains of animals, feeding and housing conditions, are important. The dose used is unimportant, however, as long as a sufficient prolongation of coagulation time is maintained for 6 days after exposure. Pichotka and Lewis state that they could not prolong the coagulation time in their animals continuously to the desired level. Their results are, therefore, of value only in the respect that they show that insufficient heparinization does not prevent gangrene after frostbite. It is interesting to analyze in detail, however, the results of Pichotka and Lewis. In their Table I, a group of 5 surviving animals received 20 mg of heparin every 4 hours after frostbite. Athough this dosage arrangement cannot be considered satisfactory according to their statements, complete necrosis occurred only in 40% of the treated animals while the controls showed complete necrosis in 79% of the animals. The other groups with longer intervals between the injections failed to show any benefit. It is astonishing to note that these facts have escaped the attention of the authors and their statisticians. The group which has the best, although not satisfactory heparinization, comprises only 12 animals while the groups with completely unsatisfactory heparinization comprise 30 and 39 animals respectively. All 3 groups are then lumped together and failure of the treatment is concluded from the average. In another paper(12), the same authors report that of 42 insufficiently heparinized animals, 76% showed no secondary infections while the non-heparinized group of 36 animals shows absence of infection in only 44% of the cases after frostbite exposure. The authors state that they are unable to explain this difference, but do not wish to attribute it to heparinization.

In order to stress the importance of a *continuous satisfactory elevation of coagulation*

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7. Lempke, R. E., and Shumacker, H. B., *Yale J. Biol. and Med.*, 1949, v21, 321; Bull. Cold Injuries and Hypothermia, National Research Council, Conf. on Cold Injuries and Hypothermia, 1948.

8. Fuhrman, F. A., and Crismon, J. M., *Clin. Invest.*, 1948, v27, 364.

9. Shumacker, H. B., *Surgery*, 1947, v22, 900.

10. Lewis, R. B., personal communication.

11. Pichotka, J., and Lewis, R. B., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 130.

12. Pichotka, J., and Lewis, R. B., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 127.

TABLE I.
Incidence of Gangrene After Frostbite in Relation to Prolongation of Coagulation Time.

Therapy	No. of animals	Complete recovery		Extensive partial or complete gangrene	
		No.	%	No.	%
Sufficient amt of heparin to keep coagulation time continuously above 30 min. for at least 5 days after exposure	34	29	85	5	15
Heparin but with occasional or common dips of coagulation time below 30 min.	17	4	24	13	76
None (controls)	32	—	—	32	100

time to prevent gangrene after frostbite, the following findings are presented.

One hind leg of each of 83 rabbits was immersed in an alcohol dry ice bath at –12°C to –30°C for 30 to 45 minutes using the technic described by us(1,4,5). Fifty-one animals were heparinized and 32 served as controls. The strain of the rabbits varied widely throughout the experiments. Small albino rabbits as used in the Friedman test were used initially and found to require extremely small amounts of heparin to maintain a sufficiently elevated coagulation time. Later, mixed strains of rabbits were used requiring widely varying amounts of heparin to obtain the desired prolongation of coagulation time. Finally, rabbits from one breeder of the Blue Vienna strain were used, giving fairly consistent results with a standardized dose of heparin. While 14 (67%) of these 21 animals showed an elevation of the coagulation time to at least 30 minutes (Lee-White method) with a standard dose immediately before the next injection, 7 (33%) fell short of this goal at one, or more frequently, several occasions on the same dose. While this is given as an example, we shall refrain from quoting doses of heparin since by injecting similar doses, similar effects on the coagulation time are not necessarily obtained. The amount of heparin must be individualized in each animal by variation of dose or preferably by variation of time interval between injections. Heparinization was accomplished by individual injections into the ear vein in 44 animals. The actual number of animals started on heparinization by repeated intravenous injections after exposure was 55. Eleven died from severe hemorrhages before the fate of the exposed leg could be determined with certainty. To avoid this difficulty (which does not exist in treatment of human cases by continuous intravenous drip) 7 rabbits were kept on a continuous intravenous drip into the ear vein while sitting in the conventional rabbit box and having one ear fixed to a splint attached upright to the upper border of the box. The flow was adjusted to a very small number of drops per minute. A feeder and a watering device were attached to the box. The rear end of the floor of the box consisted of wire mesh heavily covered with sawdust to permit drainage of urine. Only female rabbits were used in this group. Each day, the animals were taken out of the box for ½-hour, interrupting the infusion to permit revision of the dressing and to prevent contractures.

Exposures were carried out under light pentothal sodium anesthesia since the animals fight violently when exposed without anesthesia. It is difficult to understand how Pichotka and Lewis(11) were able to expose rabbits in an alcohol bath without anesthesia, since great force is needed to restrain the limbs thus producing strong local pressure and a violent alarm reaction with the probable secretion of large amounts of adrenalin. This obviously should be avoided under all circumstances.

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5. Friedman, N. B., Lange, K., and Weiner, D., *Am. J. Med. Science*, 1947, v213, 61.

All 32 control animals (exposed exactly like the heparinized animals) showed gangrene to the upper border of the exposed area. Nineteen animals were sacrificed between the 4th and 6th day to obtain specimens for histologic studies. They showed, however, clear-cut signs of incipient gangrene and the histologic studies bore out this observation. Of the 51 heparinized animals, 34 had a coagulation time of 30 minutes or more immediately before the next injection was given or, in those on the continuous intravenous drip, twice a day (Table I). Seventeen showed at one or several occasions a coagulation time below 30 minutes or even within the normal range.

Of the 34 animals showing a satisfactory elevation of coagulation time for at least 5 days, 5 developed complete or extensive partial gangrene (15%). Of the 17 animals showing a coagulation time below 30 min. at one or several occasions, 13 developed complete or extensive partial gangrene (76%).

It is thus evident that a continuous satisfactory elevation of the coagulation time of various duration depending on the severity of the lesion is a prerequisite for the successful prevention of gangrene after frostbite. Experiments in which this is not achieved are of no value in judging the merits of the method.

We suggest that the term "heparinization" be reserved only for such applications of heparin therapy where there is proof of a continuously maintained elevation of the coagulation time. The term should not be used for cases in which heparin is injected but proof of a satisfactory result is missing.

Summary. 1. Prevention of gangrene after severe frostbite requires a *continuous uninterrupted* prolongation of the coagulation time by heparin injections or infusions for at least 5 days after the exposure.

2. Even brief interruptions of this prolongation lead to a rapid increase in percentage of failure of the treatment.

3. Experiments in which such a prolongation is not achieved continuously are of no value in judging the merits of the therapy.

4. The term "heparinization" should be reserved for such instances in which heparin injections are proved to have produced a continuously maintained elevation of coagulation time to the desired level.

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Determination of Small Quantities of Nitrogen in Serological Precipitates and Other Biological Materials.* (17791)

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There exists a need for a simple and accurate method for the analysis of nitrogen in microquantities of protein, with particular application to serological precipitates. Pressman(1) and Heidelberger and MacPherson(2) have described quantitative colorimetric methods which employ the Folin-Ciocaltéu phenol reagent(3) and require an empirical calibration curve for converting the phenol color readings to nitrogen or protein. The precision and accuracy of these methods can be readily obtained by the use of Nessler's reagent, with which nitrogen is measured directly as ammonia. The method described below has been employed successfully in this laboratory for the past two years, and certain features were previously employed elsewhere (4). In its application to the analysis of serological precipitates, the chief innovation consists in that a given precipitate is prepared, washed, digested, and nesslerized in the same tube; accordingly, the errors involved in transferring precipitates are eliminated, and the time required for the analyses is reduced. At the same time, a gain in sensitivity over the previous method (4) is achieved, without sacrifice of precision, by establishing the final volume of nesslerized material at 7 ml. Since the methods for preparing serological precipitates (5) and the factors affecting the development of color with Nessler's reagent (6) have recently been discussed in detail, these matters will not be considered at length in the present communication.

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* This work was supported by a grant to Duke University from Lederle Laboratories, Inc., Pearl River, N. Y. and in part by the Commission on Influenza, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D. C.

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2. Heidelberger, M., and MacPherson, C. F. C., *Science*, 1943, v97, 405; v98, 63.

3. Folin, O., and Ciocaltéu, V., *J. Biol. Chem.*, 1927, v73, 627.

Method. Serological precipitates are allowed to form in Pyrex tubes (100 mm x 13 mm O.D.) and are washed thoroughly by routine methods (5). The tubes are drained over filter paper, and the precipitates, which should contain 5 to 80 γ N, preferably 15 to 60 γ, are treated as follows. A volume of 0.16 ml diluted H_2SO_4, prepared by mixing 1 volume concentrated acid (Mallinckrodt Low Nitrogen Analytical Reagent, specific gravity 1.84) and 1.2 volumes distilled water, is added, and the tubes are heated over a hot plate adjusted to allow refluxing of the acid about half to two-thirds of the way up the tubes. Forty-two tubes may be handled as a unit in a special circular brass rack[†] (Fig. 1) (first introduced by Prof. Dan H. Campbell in collaboration with one of us (4)). The lower plate of the rack was milled out with a ball-shaped mill to increase the area of contact with the tubes. A Meker burner may be substituted for the hot plate as source of heat. In the initial period of heating, water condenses on the cool upper portions of the tubes and may be removed by cautious application of a small flame. After one hour, the rack is allowed to cool for 4 to 5 minutes, and 1 drop 30% H_2O_2 (Superoxol, Merck) is added directly to each digest. The tubes are heated again for 5 minutes, cooled and treated with peroxide as before, and heated finally for 10 to 15 minutes. The last traces of water in the upper regions of the tubes may be removed with the use of a small flame. The tubes are then allowed to cool to room temperature.

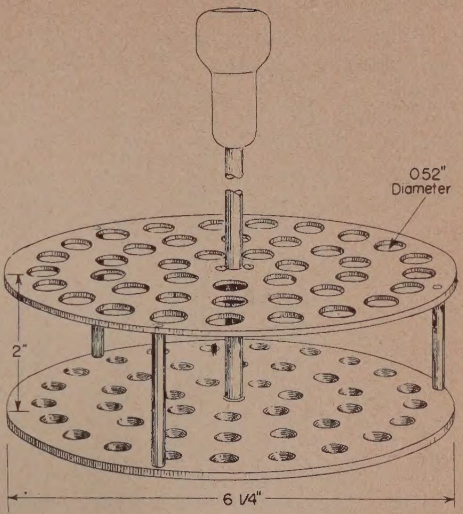

FIG. 1.

Digestion rack. The upper plate is 3/32" thick and the lower plate is 1/8". The handle is a bakelite-capped brass rod 9" long, measured from the bottom plate.

A volume of 5.00 ml distilled water is added to each tube from a buret, and the contents are mixed with a footed glass rod, which is retained. Next, 2.00 ml Nessler's reagent, prepared according to Koch and McMeekin (7)[‡], is added rapidly with a pipet, and the contents are mixed immediately with the rod, which is now set aside. A satisfactory method for delivering the Nessler's reagent

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[†] It has been found desirable to leave some of the places vacant in order to allow sufficient ventilation and thus to avoid excessive heating of the upper regions of centrally located tubes.

7. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, 1924, v46, 2066.

[‡] The 30 g mercury and 22.5 g iodine recommended by Koch and McMeekin may be satisfactorily replaced by 40.3 g mercuric iodide.

TABLE I.

Accuracy and Precision of Micro-Analytical Colorimetric (Nessler) Method as Tested with Bovine Serum Albumin (BSA) and Egg-White (EW).

Protein solution*	Date	Replicates by Nessler method, γ N			Avg, γ N	Expected,† γ N	% accounted for	Avg dev. of replicates, %
BSA-1	10/15/47	28.0	28.1	29.2	28.4	28.8	98.6	±1.8
''	''	55.8	58.4		57.1	57.6	99.1	2.3
''	11/ 1	27.1	27.5	27.6	27.4	28.8	95.1	0.7
''	''	53.9	55.6	56.7	55.4	57.6	96.2	1.8
''-2	5/ 8/48	30.1	30.9	31.1	30.7	30.9‡	99.4	1.3
''	''	61.2	62.3		61.8	61.8‡	100.0	0.9
''	10	30.2	31.1		30.6	30.9‡	99.0	1.5
''	''	60.8	62.8		61.8	61.8‡	100.0	1.6
''-3	5/12/49	32.4	32.6	33.0	32.7	33.7	97.0	0.7
''	20	52.0	52.4	53.6	52.7	56.2	93.7	1.0
''-4	20	52.9	53.8	54.2	53.6	53.4	100.4	0.9
''	25	52.8	53.4	54.0	53.4	53.4	100.0	0.7
''	6/ 2	51.2	51.6	51.6	51.5	53.4	96.4	0.3
''	10	51.2	51.6	53.2	52.0	53.4	97.4	1.5
''-5	12/ 9	18.7	19.7	20.1	19.5	20.2	96.5	2.7
''	2/ 2/50	37.2	38.2	38.7	38.0	40.5	93.8	1.5
EW-1	11/17/48	30.8	32.0		31.4	31.4	100.0	1.9
''-2	11/17/48	27.4	27.4		27.4	28.7	95.5	0.0
''-3	2/24/50	20.3	20.4	21.6	20.7	21.0	98.6	2.6
Avg							97.7	±1.4

* Aliquots of 0.50 to 2.00 ml of suitable dilutions were dried in Pyrex tubes as described in the text.

† According to determinations by semi-micro titrimetric Kjeldahl method carried out at about 1.0 mg N.

‡ 31.2 and 62.4 γ N expected according to Van Slyke gasometric determination.

consists in using a 2-ml serological pipet firmly clamped in vertical orientation to a stand. The upper end of the pipet is connected by rubber tubing to a 5-ml syringe, which is mounted at table level. The reagent is alternately drawn up into the pipet from a reservoir and emptied with moderate force directly into a diluted digest. The volume thus delivered, while not exactly 2 ml, is sufficiently constant for accurate quantitative work. The nesslerized sample is transferred to a colorimeter tube, and the optical density is determined after a timed interval, usually 60 seconds, with a suitable photoelectric colorimeter, *e.g.*, the Evelyn colorimeter, used in our work in conjunction with the No. 515 filter. Longer periods of time, at least up to one hour, may be allowed to elapse before the readings, provided that blank and standard readings are made after the same interval.

Blanks, devoid of protein, are carried along with the unknown samples through the entire process of digestion and treatment with peroxide. Some of the blanks are later converted to standards by the addition of 4.00 ml, instead of 5.00 ml, H_2O and 1.00 ml standard $(NH_4)_2SO_4$ solution (preserved with 1.4 ml concentrated H_2SO_4 per liter) containing 5 to 80 γ N per ml. A standard curve, covering the expected range of nitrogen values, is prepared with each run. Such standard curves, in which optical density is plotted against quantity of nitrogen, are sufficiently linear that they may be regarded as such in the evaluation of the results(6). The above method is readily adapted to the analysis of protein solutions by the simple expedient of drying down a suitable volume of solution at 90 to 95°C in a Pyrex tube of the size indicated above.

Results. Table I illustrates the accuracy and precision of the method. Stock solutions of bovine serum albumin and egg-white, containing about 1.0 mg N per ml, were analyzed by a semi-micro titrimetric Kjeldahl method to provide reference values. Aliquots of suitable dilutions were analyzed by the colorimetric method. On one occasion a stock solution of bovine serum albumin was analyzed also by a Van Slyke gasometric procedure. The colorimetric method yielded, on the average, 97.7% of the nitrogen expected from

TABLE II.
Precision of Determination of Amounts of Nitrogen Precipitated from Duplicate Mixtures of Purified Swine Influenza Virus and Convalescent Swine Influenza Antiserum.

Duplicates γ N		Avg dev., %	Duplicates, γ N		Avg dev., %
4.0	4.5	±5.9	21.0	21.8	±1.9
6.0	6.4	3.2	22.1	23.4	2.9
7.0	7.5	3.4	23.0	23.9	1.9
7.4	8.7	8.1	24.0	24.6	1.2
8.6	8.8	1.1	24.0	25.3	2.6
10.2	10.6	1.9	25.0	25.4	0.8
9.8	11.5	8.0	25.1	25.3	0.4
12.8	13.1	1.2	26.6	27.0	0.7
13.9	14.1	0.7	27.4	27.4	0.0
12.8	15.3	8.9	27.3	27.9	1.1
14.6	14.9	1.0	27.3	28.0	1.3
15.2	16.2	3.2	26.8	28.9	3.8
15.4	16.2	2.5	27.3	28.4	2.0
15.6	16.1	1.6	30.7	31.4	1.1
15.8	17.2	4.2	34.6	35.0	0.6
17.1	17.4	0.9	35.8	37.5	2.3
17.1	19.5	6.6	39.3	40.2	1.1
18.4	20.0	3.2	45.8	46.2	0.4
20.4	20.4	0.0	45.0	49.3	4.6
20.0	21.1	2.9	50.0	50.8	0.8

the titrimetric analysis; the average deviation of replicate determinations was about ± 1.4%. The yield with respect to the Van Slyke procedure was 98.6%. Investigation showed that approximately 1% of the digest was lost by spattering during each of the 2 treatments with peroxide. With a correction for these losses, the accuracy of the microanalysis closely approaches 100%.

The precision of the method for the determination of nitrogen in serological precipitates is illustrated in Table II, which shows results obtained with washed precipitates from mixtures of purified swine influenza virus and convalescent swine influenza antiserum. The errors in these analyses arise from the formation and handling of the precipitates, as well as from the nitrogen determination. It is evident that the overall precision is compatible with the demands of quantitative serological investigations. Results similar to those of Table II have been obtained with mixtures of SII, the type-specific capsular polysaccharide of Type II pneumococcus, and homologous horse antiserum.

The method is not intended to replace the more precise and accurate titrimetric methods, but is regarded as useful for the analysis of small samples, costly samples, or large numbers of routine samples(8-11) whenever the indicated errors can be tolerated. Experience has shown that its useful range extends from about 5 to 80 γ N.§

Summary. A simple and accurate colorimetric method is described for the routine determination of quantities of nitrogen in the range from 5 to 80 γ. The method, which is a modification of the direct-nesslerization procedure of Koch and McMeekin, has been developed especially for the analysis of serological precipitates, but is adapted readily to the analysis of protein solutions.

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§ The authors are grateful to Mrs. Edith S. Dillon for assistance.

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Hyperglycemic Properties of Crude Pancreatic Proteins.* (17792)

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The presence of a factor in crude insulin that produces a transitory hyperglycemia when injected intravenously has long been known(1). Its presence was reported in amorphous insulin and certain commercial preparations of crystalline zinc insulin(2,3), and in the supernatant of the first isoelectric precipitation of insulin(4). These observations prompted us to test pancreatic fractions that were obtained in the course of another investigation(5) from the supernatant of the 15% sodium chloride precipitate of the crude insulin salt-cake prepared by a slight modification of the procedure of Romans, Scott and Fisher(6). To those engaged in the isolation of this factor, its presence in the 15% sodium chloride supernatant and observations on the intravenous and subcutaneous administration of these fractions may be of interest.

Experimental. The procedure employed for the preparation of the pancreatic fractions from the 15% NaCl supernatant has been described in another publication(5), and is briefly outlined in flow sheet form in Table I. All preparations were dialyzed thoroughly against running tap water for at least 48 hours, filtered through clarifying pads (Republic K-3) and then lyophilized. The alkaline treatment described by Heard and coworkers(4) was employed to inactivate insulin.

Blood glucose was determined by the official U.S.P. XIII method(7). As a rule, each preparation was tested on a group of 5 or 6 rabbits (2 kg), and the results for each bleeding were averaged. To eliminate the variations in absolute blood sugar levels, the average blood sugars for each bleeding period were expressed in terms of percentage of the average preinjection blood sugar value.

Results and discussion. Hyperglycemic activity of pancreatic fractions. With the exception of No. 3 all preparations tested produced a mild hyperglycemia of short duration, followed by a marked lowering of blood sugar which in a few animals resulted in mild convulsions (Fig. 1). The nature of this hypoglycemic response indicated the apparent presence of about 1 unit of insulin. Only the trichloracetic acid insoluble fraction (No. 3) apparently was free of insulin.

It was evident that in preparations containing insulin, the full effects of the hyperglycemic factor were masked. Inactivation of the insulin in these fractions† with alkali(4) yielded preparations which showed no evidence of insulin (Fig. 1), though it is possible that the effects of small amounts of it might have been overshadowed by the hyperglycemic factor.

The alkali-treated preparations and the "non-treated" preparation, No. 3, were all essentially equally active in raising blood sugar levels. Similar results were obtained with two other series of fractions (tested at dosage levels between 1 and 10 mg/kg), one of which was treated with alkali before fractionation. The presence of the hyperglycemic factor in almost all fractions indicated lack

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* Presented in part at the American Chemical Society meetings Jan. 20, 1949, Philadelphia, Pa.

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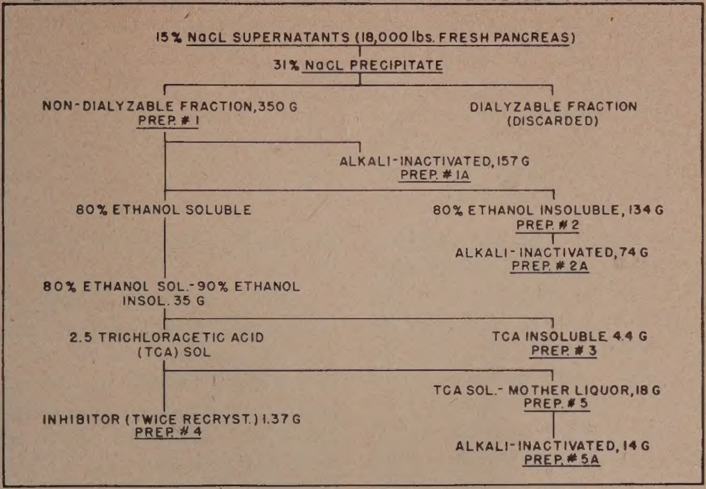
† Due to lack of an adequate supply of the crystalline inhibitor (No.4), this preparation was not treated with alkali.

TABLE I
PREPARATION OF PANCREATIC FRACTIONS WITH HYPERGLYCEMIC PROPERTIES


15% NaCL SUPERNATANTS (18,000 lbs. FRESH PANCREAS)
31% NaCL PRECIPITATE
NON-DIALYZABLE FRACTION, 350 G
PREP. # 1
DIALYZABLE FRACTION
(DISCARDED)
ALKALI-INACTIVATED, 157 G
PREP. # 1A
80% ETHANOL SOLUBLE
80% ETHANOL INSOLUBLE, 134 G
PREP. # 2
ALKALI-INACTIVATED, 74 G
PREP. # 2A
80% ETHANOL SOL.-90% ETHANOL INSOL. 35 G
2.5 TRICHLORACETIC ACID (TCA) SOL.
TCA INSOLUBLE 4.4 G
PREP. # 3
TCA SOL.- MOTHER LIQUOR, 18 G
PREP. # 5
INHIBITOR (TWICE RECRYST.) 1.37 G
PREP. # 4
ALKALI-INACTIVATED, 14 G
PREP. # 5A

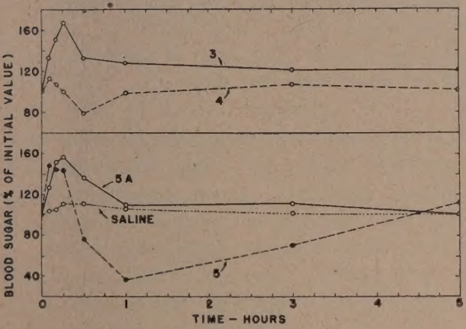


FIG. 1.

Effect on blood sugar levels of rabbits receiving pancreatic fractions before and after alkaline inactivation of insulin. Curves marked "A" were obtained with alkali-treated preparations. The numbers refer to preparations in Table I. Each curve is the average of 5 or 6 rabbits. The preparations were injected intravenously 20 mg/kg except No. 4 (10 mg/kg) and saline (0.5 ml/kg). Preparations No. 1 and No. 2 produced a biphasic glycemic effect similar to curve 5 except that the hyperglycemia was less marked and of shorter duration. Preparations No. 1A and No. 2A produced a hyperglycemic response almost exactly identical with that of preparation No. 5A.

of adequate separation of this factor from contaminating substances and suggested that it possibly was being readily adsorbed by the protein(s) in these fractions. The presence of the factor in crystalline zinc insulin and amorphous insulin(2,3), in the supernatant of first iso-electric precipitate(4) and now in the 15% NaCl supernatant and the various fractions derived from it support this possibility. Except for the separation of the factor from insulin by 2.5% trichloracetic acid (preparation No. 3), it is doubtful whether any degree of purification was effected.

Quantitative aspects. The wide variation in response among individual rabbits to the injection of the hyperglycemic factor, especially evident at lower dosages, suggested that the test employed was not suitable for quantitative studies. An analysis of variance on the data obtained (Table II) indicated the log-dose-response curve to be linear over the range covered by the 4 lowest doses. However, the slope of the regression line was too low for statistically significant results without employing an impractically large number of animals at each dose level. Therefore it was concluded that the hyperglycemic effects produced under the specified conditions could be interpreted only qualitatively.

Subcutaneous administration. When the hyperglycemic factor was injected subcutaneously in single dosages of 100 and 200 mg/kg it produced blood sugar levels as high as 225% of the initial value in some rabbits. Maximum levels were reached in approximately 30 minutes.

TABLE II.
Dose-response Effect of Hyperglycemic Factor (Prep. No. 1A) Administered I.V. in Rabbits (Groups of 6 Animals).

Blood sample (min.)	Dose (γ/kg)							
	0*	5	20	80	100	300	900	2700
10	95†	98	105	110	140	130	130	145
20	100	103	112	114	137	120	123	142

* Distilled water—0.5 ml/kg.
† Blood sugar—% of initial value.

In some of the animals multiple subcutaneous injections (Fig. 2) raised the blood sugar level above 300% of the initial value. The administration of 0.75 units of crystalline Zn insulin per kg with the initial dose did not significantly change this response. This amount of insulin is sufficient to cause a 50% or greater reduction in blood sugar level within 2 hours, and to produce convulsions in the more sensitive animals. When insulin and the hyperglycemic factor were administered together, neither hypoglycemia nor convulsions were observed during this period. However, continued administration of the hyperglycemic factor, with or without the initial dose of insulin, produced a convulsive and fatal level of blood sugar after 7 hours. Heard *et al.*(4) and Burger and Kramer(9) have eliminated the possibility that this factor produces its effect through a non-specific action or adrenal or anterior pituitary intervention. Therefore the continued administration of large amounts of this factor probably produced a depletion of the glycogen reserves of the liver.

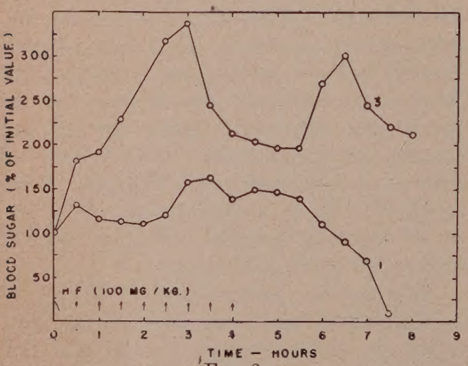


FIG. 2

Effect on blood sugar levels of rabbits receiving multiple subcutaneous injections of 100 mg/kg of Preparation No. 1A at half-hourly intervals. Each curve represents a single animal.

Stability in blood; hyperglycemic property of incubated blood. Sutherland and de Duve (8) were unable to identify with certainty the hyperglycemic factor in citrated plasma collected from the pancreatic duodenal vein, and found that when the factor was added to blood it was destroyed at an appreciable rate. Our factor apparently was not destroyed when incubated at 37°C for 1 hour with heparinized whole blood since the injection of the blood produced a hyperglycemic effect like that produced by the factor alone. No significant increase in blood sugar was observed in animals injected with blood incubated under the same conditions, but without the factor. In our experiments, perhaps the presence of heparin accounted for the stability of the hyperglycemic factor.

A secondary rise in blood sugar did occur in 1 to 3 hours in control animals injected with blood held for one hour at 37°C. This was confirmed in other experiments (Fig. 3) in which heparinized blood injected immediately after collection from donor rabbits did not produce any increase in blood sugar, but incubated blood did. Hence incubation of such blood apparently released a substance which had hyperglycemic properties or which acted through the adrenal or anterior pituitary glands or in some other way. Obviously these experiments afford no explanation of mechanism of action.

Protection of mice from insulin shock. The observation(9) that the hyperglycemic factor

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8. Sutherland, E. W., and de Duve, C., *J. Biol. Chem.*, 1948, v175, 663.

9. Zimmermann, B., and Donovan, T. J., *Am. J. Physiol.*, 1948, v153, 197.

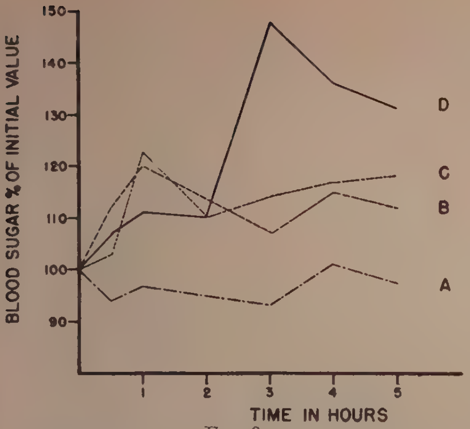

Fig. 3

Effect on blood sugar levels of rabbits receiving 10 ml injections of heparinized blood. Each curve represents average of 3 animals except C (2 animals). Curves A, B, and C were obtained by injecting pooled heparinized blood taken from several donor rabbits: A—blood injected immediately (within 10 min) after collection, B—after incubation at room temp for 1 hr, and C—after incubation 37°C for 1 hr. Curve D by reinjecting the animals' own heparinized blood after incubation 37°C for 1 hr.

does not protect mice against insulin-induced shock was confirmed by thoroughly controlled experiments with groups of 30 and 40 mice receiving 100 mg/kg (single and multiple doses) of the hyperglycemic factor at various intervals before and/or after the injection of insulin (0.016 unit/mouse). Failure of relatively large doses to protect mice against insulin-induced convulsions seems to be at variance with the results obtained in rabbits, in which the subcutaneous injection of the hyperglycemic factor in large doses successfully reversed the hypoglycemic effect of the insulin. The absence of data on the actual blood sugar levels in the mice makes interpretation of the results difficult.

Summary. The hyperglycemic activity of certain fractions derived from the 15% sodium chloride supernatant, a by-product of insulin manufacture, was determined before and after alkaline inactivation of the insulin present in these preparations. Hyperglycemic activity was studied in the rabbits but the results were interpreted only qualitatively, since a satisfactory log dose-response was not obtained. The factor was active whether intravenously or subcutaneously administered. Repeated subcutaneous administration produced hypoglycemia and convulsions in some animals. The factor appeared to be stable in heparinized blood at 37°C for 1 hour; incubated blood itself produced a delayed hyperglycemic effect when injected intravenously. The factor did not protect mice against insulin-induced convulsions, but did reverse the hypoglycemic effect of insulin in the rabbit.

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Stimulatory Effect of Aureomycin on the Growth of Chicks. (17793)

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Several reports in the literature suggest that the growth-promoting activity of "animal protein factor" concentrates for chicks is not due solely to the vitamin B_{12} content of such supplements(1-3). Stokstad *et al.*(4) in studying the distribution of this additional factor(s) found that fermentation liquors of *Streptomyces aureofaciens* were capable of producing growth increments in chicks greater

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1. Menge, H., Combs, G. F., and Shorb, M. S., *Poultry Sc.*, 1949, v28, 775.

2. Carlson, C. W., Miller, R. F., Peeler, H. T., Norris, L. C., Hauser, G. F., *Poultry Sc.*, 1949, v28, 750.

3. Hill, F. C., *Poultry Sc.*, 1948, v27, 536.

4. Stokstad, E. L. R., Jukes, T. H., Pierce, J., Page, A. C., and Franklin, A. C., *J. Biol. Chem.*, 1949, v180, 647.

TABLE I.
Comparison of Aureomycin HCl to Various Supplements with APF Deficient Chicks.

Supplements to Diet I kilo of diet	Exp. 1		Exp. 2		
	3 wk, g	4 wk, g	3 wk, g	4 wk, g	P
None	235	320(10)	207	301(8)	
25 cc. *S. aureofaciens* mash	257	390(10)			.01
50 " " " "	243	373(10)			.01
100 " " " "	235	348(10)			.02
10 mg aureomycin HCl	264	367(10)			.01
25 " " "	273	407(10)			.01
50 " " "	262	377(10)			.01
100 " " "	279	385(9)	241	358(10)	.01
200 " " "	269	380(10)			
100 " streptomycin			240	351(9)	.01
100 " chloromycetin			214	319(8)	.05
100 " penicillin			249	361(10)	.01
100 " sulfasuxadine			222	321(9)	.05

P value calculated by the simple rank test using unpaired replicates.
No. of chicks in parentheses.

than those obtained with vitamin B_{12} alone. Cunha *et al.*(5) obtained the same results with swine fed this fermentation APF supplement. As aureomycin is a known constituent of *S. aureofaciens* liquors, this compound was tested and found to possess a marked ability to stimulate the growth of the chicks. Other antibiotics elicited a response under similar conditions.

Experimental. The APF deficient chicks used were Barred Rock New Hampshire Red crosses from a laying flock which was maintained on the APF deficient diet and under the conditions described by Stokstad *et al.*(4). The hens had been fed the deficient diet for approximately 3 months before the progeny were used in these experiments. The normal chicks were New Hampshire Reds purchased from a commercial hatchery. The day old chicks were housed on raised screens in heated brooders and were fed the experimental diets and water *ad libitum.* Since all diets were supplemented with excess vitamin B_{12}, survival of chicks fed these diets was usually 100%. Growth was used as a measure of activity. All supplements were incorporated in the basal diets. Diet I is the corn-soybean diet for chicks reported by Stokstad *et al.*(4). Diet II is the synthetic diet of Oleson *et al.*(6). supplemented with 2 mg of pteroylglutamic acid per kilo. Diet III differs from diet II in that the casein and gelatin are replaced by 22% α protein and 0.3% methionine. All diets were supplemented with 90 γ of vitamin B_{12} per kilo.

Results. The comparative growth-promoting activity of whole *S. aureofaciens* mash, aureomycin hydrochloride and certain other antibiotics is shown in Table I. The data indicate that aureomycin at a level of 25 mg per kilo gives the same stimulatory effect as whole *S. aureofaciens* mash. Certain other antibiotics were tested at levels of 100 mg per kilo. Of these only penicillin gave a response equivalent to that produced by aureomycin.

The growth-promoting effect of aureomycin is not limited to APF deficient chicks or to chicks fed the corn-soybean diet. The results presented in Table II indicate that the growth of normal hatchery run chicks fed several different diets, including a commercial chick starter mash, is significantly increased by the inclusion of aureomycin in the feed.

In two experiments, when graded levels of aureomycin or penicillin were injected intravenously or intramuscularly, no significant growth responses over the control group were obtained.

Discussion. The data presented indicate that the inclusion in the diet of aureomycin

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5. Cunha, T. J., Burnside, J. E., Buschman, D. M., Glasscock, R. S., Pearson, A. M., Shealy, A. L., *Arch. Biochem.*, 1949, v23, 324.

6. Oleson, J. J., Hutchings, B. L., and Sloane, N. H., *J. Biol. Chem.*, 1946, v165, 371.

TABLE II.
Effect of Aureomycin on Growth of Normal Chicks on Various Diets.

	Wt at 3 wk							
Supplement	Diet I, g	P	Diet II, g	P	Diet III, g	P	Commercial chick starter, g	P
None	242(10)		223(9)		211(10)		195(10)	
50 mg aureomycin	273(10)	.05	263(10)	.05	240(10)	.05	226(10)	.02

P value calculated by the simple rank test using unpaired replicates.
No. of chicks in parentheses.

or certain other antibiotics will produce a growth stimulation greater than that obtained by vitamin B_{12} alone. The activity of these compounds is presumably related in some way to their antibiotic activity. The lack of activity of intravenously administered aureomycin or penicillin is consistent with this hypothesis. However, the mode of action of these antibiotics on the intestinal flora remains to be determined. Moore *et al.*(7) have previously reported the stimulatory effect of streptomycin on the growth of the chick.

A number of source materials have been reported to possess growth-promoting activity greater than could be accounted for on the basis of their vitamin B_{12} content(8-11). Since these natural materials are not known to contain any antibiotics, the relation of the effect of these materials to the growth stimulation induced by aureomycin is difficult to assess at this time. It may be possible that the factor present in the natural supplements and the factor presumably produced by the action of aureomycin in the intestinal flora of the chick are similar.

Summary. Aureomycin hydrochloride stimulates the growth of chicks when fed in the diet at levels as low as 25 mg per kilo. This effect is presumed to be an indirect one since certain other antibiotics manifest this same property. The relationship of this phenomenon to proposed new APF factors in certain natural materials is discussed.

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8. Hartman, A. M., Dryden, L. P., and Cary, C. A., *Proc. Am. Chem. Soc.*, Sept. 1949, p. 39A.

9. Carlson, C. W., Combs, G. F., Miller, R. F., Yacowitz, H., Norris, L. C., and Scott, M. L., *Proc. Am. Chem. Soc.*, Sept. 1949, p. 37A.

10. Ansbacher, S., and Hill, H. H., *Proc. Am. Chem. Soc.*, Sept. 1949, p. 31A.

11. McGinnis, J., Stephenson, E. L., Levadie, B. T. H., Carver, J. S., Garibaldi, J. A., Ijichi, K., Snell, N. S., and Lewis, J. C., *Proc. Am. Chem. Soc.*, Sept. 1949, p. 42A.

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Alveolar Ventilation Studies Using the Mass Spectrometer.* (17794)

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In recent investigations the rate of intrapulmonary mixing of gases has been used as a measure of respiratory function. Cournand *et al.* examined the intra-alveolar mixing of tidal air by determining the concentration of nitrogen in an alveolar sample after the subject respired 100% oxygen for 7 minutes. Alveolar ventilatory insufficiency was as-

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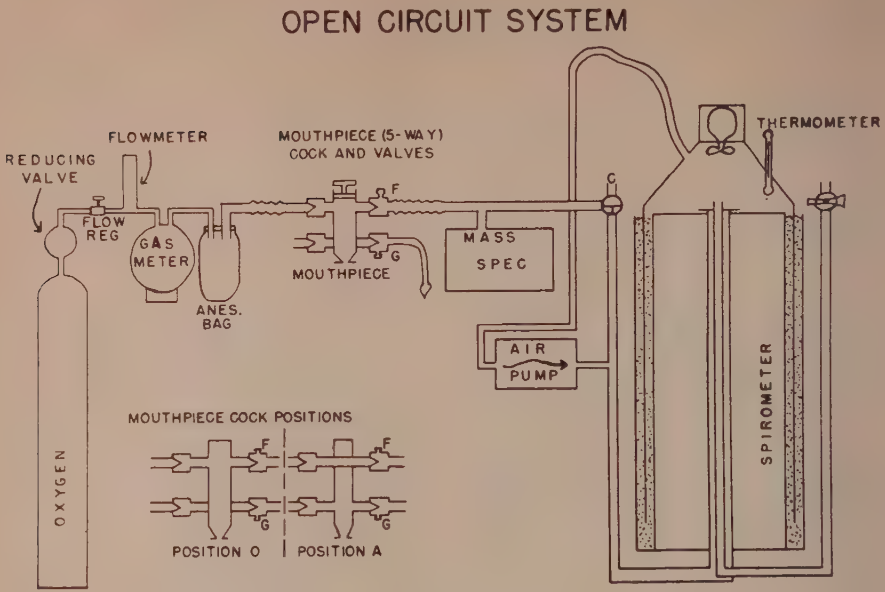

Fig. 1.
Schematic diagram of apparatus used for nitrogen elimination test.

sumed to be present when this value was greater than 2.5%; this situation obtained in 97% of emphysematous patients(1). The time required for chemical nitrogen analysis in their studies limited the value of this pulmonary nitrogen clearance test, for but one point on the elimination curve was determined. Meneely and Kaltreider sought to resolve this difficulty with a catharometer(2). The analytical cell had, however, a marked lag period and therefore made the values relative rather than absolute. One of us, (A.O.C.N.), recently developed a portable mass spectrometer capable of continuous analysis of five different gases every 20 seconds with an accuracy of approximately 1%. This device is being used to test the efficiency of alveolar ventilation as evidenced by nitrogen elimination curves breathing 100% oxygen. With this instrument it is hoped that the above indicated limitations have been largely abolished.

Method. With the subject in a sitting position, a 5 way valve is connected to a rubber mouthpiece and a conventional nose clip is applied. The diagrammatic representation of the valve and this set up are shown in Fig. 1. After an equilibration period breathing air, the valve is turned precisely at the end of a normal expiration, and the subject then inspires 100% oxygen from a partially inflated anesthetist's breathing bag. The expired mixture is discharged through the mass spectrometer and minute amounts are removed for analysis. The remainder of the expired gas flows into a 100 liter Tissot spirometer and the volume and temperature are recorded. When the mass spectrometer records a nitrogen concentration near equilibrium, the test is concluded and the subject is switched to breathing air. Values from this nitrogen regression curve are now plotted against the minute volume of the expired gas mixture, corrected to 37°C, saturated water vapor tension and standard barometric pressure.

Fig. 2 depicts a mass spectrometer record during a nitrogen elimination test in a normal individual. In this instance the nitrogen clearance was "complete" after 5 minutes of

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1. Cournand, A., Baldwin, E. DeF., Darling, R. C., and Richards, D. W., *J. Clin. Invest.*, 1941, v20, 681.

2. Meneely, G. R., and Kaltreider, N. L., *J. Clin. Invest.*, 1949, v28, 129.

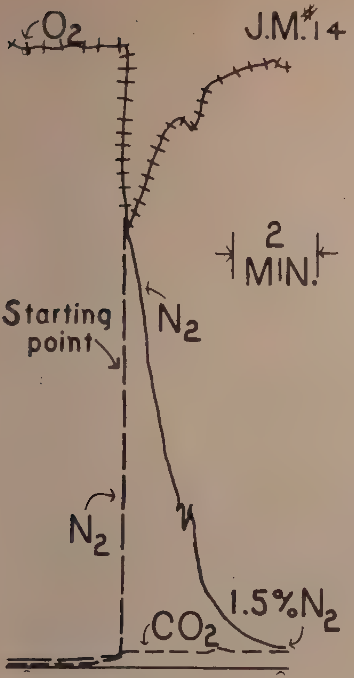

Fig. 2.
Mass spectrometer recording during a nitrogen elimination test on a normal individual.

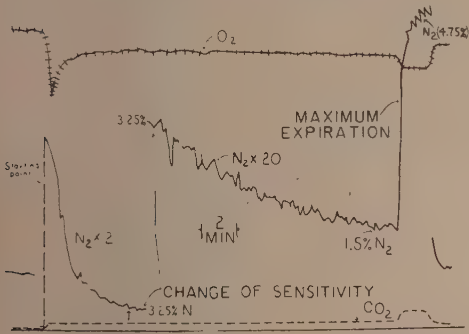

Fig. 3.
Mass spectrometer tracing of a nitrogen elimination test on an individual with marked emphysema. In this recording the sensitivity of the machine was changed during the test in order to determine at what point, if any, the curve leveled off.

100% oxygen inspiration. The asymptotic nature of the curve is due to the nitrogen excreted into the lungs from the blood, a very slow process. Fig. 3 in contrast shows the curve of an emphysematous individual. Note that at the end of 20 minutes the clearance is still incomplete as demonstrated by the marked rise in the nitrogen value following a maximal expiration. In normal alveoli, which are virtually free of nitrogen, the rise after this maneuver is but a fraction of a per cent.

To avoid the errors imposed by changes in respiratory minute volume, all nitrogen elimination curves have been corrected to nitrogen clearance volume. This is by definition the quantity of diluting gas required to reduce the nitrogen content of the expired air to no more than 1% greater than the contaminant concentration of nitrogen in the diluting gas.

Eighteen clearance determinations were performed on 5 normal adult male students. Table I shows the results of multiple determinations on a single individual. The clearance volumes appear to be quite reproducible, with 4 liters the maximum deviation for one individual, and in the remainder duplicate tests agreed within 2.4 liters, a volume equivalent to 5 normal expirations. In comparing the different individuals, there is a maximum difference of 16.8 liters between 2 of the normal subjects. It is possible to correct this by correlation with total lung volume measurements(3).

The remaining portion of Table I shows the nitrogen clearance volumes of 3 patients with marked clinical pulmonary emphysema. A significant difference between the emphysematous individuals and the normal controls is apparent. The assumption that these patients are suffering from an inability to ventilate adequately the alveolar portions of their lungs seems valid.

Summary. A method of measuring alveolar ventilation utilizing a portable mass spectrometer to determine the rate of nitrogen elimination is described. This test was shown by repeated trials to be rather uniform for each of a group of normal individuals. The range of nitrogen clearance volumes among normal individuals appears to be related to differences in lung volume, and correction for this factor approximates the pattern of values

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3. Miller, F., Hemingway, A., Nier, A. O. C., and Varco, R., to be published.

TABLE I.
Nitrogen Clearance Data from 5 Control Subjects and 3 Patients with Marked Emphysema.

Subject	Condition	Height, cm	Body wt, kg	N_2 clearance vol., l
D.	Normal	283	63.5	32.8
				32.8
				32.
				32.
J.M.	"	268	66.0	29.2
				27.6
				27.6
J.S.	"	260	59.2	26.8
				26.
				22.8
M.	"	280	75.0	22.8
				22.8
				20.4
				20.4
B.	"	280	77.4	17.6
				17.3
				16.8
				16.
L.	Emphysema	280	63.5	116.
K.	"	264	61.0	120.
A.	"	264	77.5	160.

for those tested. Three cases of marked pulmonary emphysema are presented. The results from testing this group in a similar fashion indicate a prolongation of nitrogen elimination in these patients as compared with the normal subjects.

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Cross Transfusion. II. Therapeutic Effect in Acute Mercury Nephrosis.* (17795)

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Recently, a safe and controllable method for cross transfusion and the instrument for this purpose have been described(1). The difficulties and dangers of earlier methods (2-7), which often caused the death of experimental animals, were encountered when the experiments were repeated by us. Briefly, the difficulties have been:

1. Sacrifice of an artery to obtain blood (2-7). This can be repeated only a few times in the same individual. It is also unsuitable because of the occurrence of arterial spasm, which often terminates the procedure, and because withdrawal of blood from an artery against a low resistance puts an additional load on the heart. Methods which alternately withdraw and administer blood often cause shock(3).

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* This study was supported in part by a grant-in-aid from Mr. Emil Friedlander, and by a grant to Dr. Goldblatt from the Los Angeles Heart Association.

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2. Duncan, G. G., Tocantins, L., Cuttle, T. C., PROC. SOC. EXP. BIOL. AND MED., 1940, v44, 196.

3. Green, H. D., *Rev. Scientific Instruments*, 1945, v16, 95.

4. Little, J. M., Green, H. D., Hawkins, J. E., *Am. J. Physiol.*, 1947, v151, 554.

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6. Thalhimer, W., Solandt, D. Y., Best, C. H., *Lancet*, 1938, v235, 554.

7. Green, H. D., Bergeron, G. A., Little, M. J., and Hawkins, J. E., *Am. J. Physiol.*, 1947, v149, 112.

TABLE I.
Determination of the MLD_{100} of Intravenous $HgCl_2$ in Adult Dogs.

Wt, kg	Sex	Dose $HgCl_2$ mg/kg	Last measured plasma creatinine level mg/100 cc	Survival, days
12.3	M	5.	8.90	1.96
17.5	F	4.	14.90	2.87
16.8	F	3.5	12.89	3.54
18.2	F	3.5	9.20	1.83
20.5	M	3.	7.78	3.50
15.4	M	2.5	8.84	5.00
22.3	M	2.5	20.03	8.75
15.9	M	2.5	6.98	2.62
18.2	M	2.5	21.40	8.13
18.2	M	2.5	10.64	3.41
21.4	M	2.	22.40	8.62
18.1	M	2.	16.06	6.00
10.5	M	2.	15.48	9.76
10.9	F	2.	15.48	8.08
10.5	F	2.	17.20	3.66
15.0	M	2.	1.03	4.50
21.0	F	1.5	1.79	S
5.0	M	2.	.60	S
9.5	F	2.5	.62	S

S—Survived.

2. Roller pumps and worm pumps (6) often cause hemolysis in a ratio of about .1% per single passage of blood through the apparatus. This is a toxic amount in many cases.

3. Clotting and deposition of proteins occur in spite of the use of anticoagulants(6). This slows down and often stops the flow of blood. It also interferes with the metering function of roller pumps because it narrows the blood conduits.

4. The absence of accurate measuring devices for blood flow. A small error becomes magnified over a period of time, leading to oligemia in one organism and plethora in the other, often with fatal results(2-7).

Our apparatus does not injure the blood and withdraws and administers blood continuously and simultaneously through double lumen intravenous catheters. It pumps and measures with a high degree of accuracy the blood flowing in both directions, and automatic electric devices adjust the flow. In order to evaluate the effectiveness of cross transfusion it was decided to study a pathological condition which was easily reproduced, well-known, and capable of quantitative analysis. The intravenous administration of a 100% lethal dose of mercuric chloride was chosen as the test condition.

Methods and procedure: Adult mongrel dogs, varying in weight between 10½ and 23 kg, were used. The mercuric chloride was administered as a 5.00 mg/cc solution in physiological saline given rapidly into the external saphenous vein. Arterial heparinized blood samples were taken at the time of injection and at daily or 48 hour intervals thereafter. Hematocrit determinations of the centrifuged specimens were made and the plasma creatinine was determined by the method of Bonsnes and Tausky(8). Autopsies were performed and the organs preserved in 10% formalin for histological study. Several animals died from causes not attributable to mercury nephrosis or to cross transfusion and are not included here. The following procedure was used: the jugular or femoral vein of each animal was isolated, under local anesthesia, and a double lumen catheter passed cardiad for a distance of about 15 cm. About 3.75 mg of heparin per kg of body weight was injected through the catheter and blood samples were withdrawn. The catheters were then attached to the apparatus which previously been filled with 230 cc of heparinized saline and the cross transfusion started at a rate of 5 to 6 liters an hour in each direction. At the end of the cross

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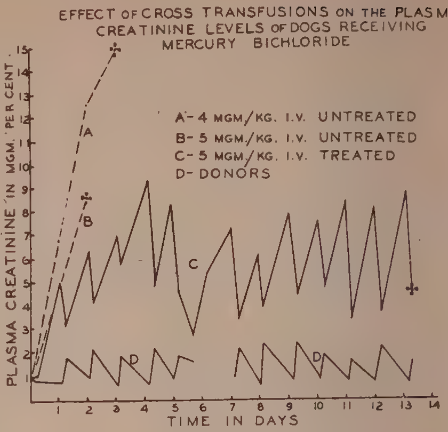
8. Bonsnes, R. W., and Tausky, H. H., *J. Biol. Chem.*, 1945, v158, 581.

transfusion, samples of blood were again taken and the blood in the apparatus was washed into the animals by 100-200 cc isotonic solutions. Additional heparin was added occasionally during the procedure. Animals received sedation in some of the experiments, which had no effect on the results. Cross matching, when done, had no appreciable effect on the outcome of the experiment. Sterile technic is desirable. 150,000 U of procaine penicillin in oil was given as a precautionary measure. The duration of the cross transfusions and the quantity of blood transferred could be varied at will.

Results and discussion. 1. *Determination of the minimum 100% lethal dose of intravenous $HgCl_2$ in the dog* was made by means of 19 animals (Table I).

All the animals weighing more than 10.5 kg that received 2.0 mg $HgCl_2$ or more per kg of body weight died within 10 days. 2.5 mg/kg was chosen as the minimum LD100 for full grown dogs weighing 10.5 kg or more, when mercuric chloride is injected rapidly by the intravenous route. This value is not applicable to very young dogs or dogs weighing less than 10.5 kg, as is illustrated in the last two entries in Table I. The textbooks give the figure of 4 mg/kg as the intravenous MLD100 of $HgCl_2$ for the dog. The experiments on which this figure is based were performed by Sansum(9) who gave intravenous injections to 3 dogs "by means of a special pump, so that each injection took exactly thirty minutes to accomplish". This method of injection is different from the customary intravenous injection, which is performed within a few seconds, and it is not surprising, therefore, that Sansum's MLD100 is different from ours.

2. *Evidence of Therapeutic Efficacy of Cross Transfusion.* (a) *Transfusion begun 1-2 days after poisoning with mercuric chloride.* In 5 experiments, animals poisoned from 1-2 days before the first transfusion, were treated repeatedly at intervals of 24 to 120 hours by cross transfusions, lasting 2 to 5 hours, with presumably normal donors. In every case the animal was maintained alive for a time 2 to 3 times longer than controls receiving the same dose of $HgCl_2$. One animal which had received 2.5 mg $HgCl_2$ per kg, recovered after 2 cross transfusions. Graph I represents the creatinine levels in one of these experiments and is also illustrative of the others. This animal received 5.0 mg/kg of $HgCl_2$ which was twice the MLD. Each abrupt drop in the plasma creatinine level of the patient is the result of a cross transfusion. At postmortem examination the epithelium of the tubules of both kidneys was completely necrotic and in great part, calcified, without any evidence of regeneration.



Graph 1.

The donors showed no signs of injurious effects. Their plasma creatinine levels returned to normal well within 24 hours. The occasional chills, retching, vomiting, and, in two cases, hives, disappeared soon and did not constitute cause for discontinuance of the experiment. Catherized urine specimens did not contain abnormal amounts of protein. It can be observed that the creatinine level of the patient's blood usually dropped more than it rose in the donor's blood. This difference is taken to illustrate the lowering of the plasma creatinine level because of excretion by the donor's kidneys.

(b) *Transfusion begun 1-3 hours after poisoning.* Six animals were given 2.5 mg $HgCl_2$ per kg and then transfused either one

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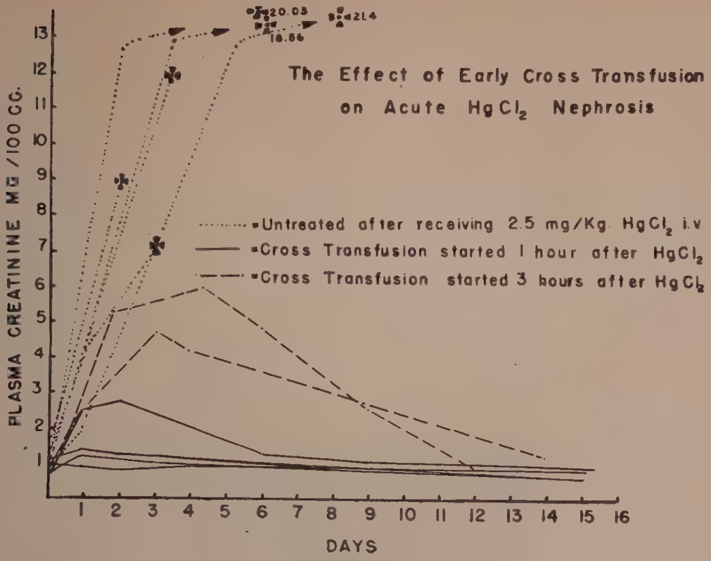
9. Sansum, W. D., *J. A. M. A.*, 1918, v70, 824.



GRAPH II.

hour or 3 hours after the administration of the mercury. Graph II shows the plasma creatinine levels of the animals so treated. The 4 dogs treated at the end of 60-90 minutes remained clinically well and did not show any significant or prolonged elevation of plasma creatinine. Pathological changes were not found on microscopic examination of the kidneys 3 to 6 weeks later. The 2 dogs cross transfused 3 hours after the administration of the $HgCl_2$ showed clinical evidence of mercury poisoning, such as anorexia, vomiting, diarrhea, lassitude and also transitory elevation of blood creatinine, but recovered within 10 days.

The mechanism of action of cross transfusion in preventing a fatal outcome in these animals is not known. We believe that cross transfusion serves to reduce the amount of mercury in the poisoned animal to a sublethal quantity by partial transfer to the donor. As indicated by the discrepancy between Sansum's and our figure for the MLD 100, the donor would not be harmed, because the mercury is received by the donor gradually over a period of time and already partly detoxified. The transfer of detoxifying substances to the poisoned animal from the donor may also play an important part.

Summary. 1. When performed with inadequate equipment, cross transfusion may be a dangerous procedure. However, it has been shown in experiments on dogs, that with proper apparatus and technic, cross transfusion is safe for both donor and patient.

2. For dogs weighing 10.5 kg or more, the intravenous MLD100 of mercuric chloride is 2.5 mg/kg.

3. Cross transfusion is an effective means for lowering the non-protein nitrogenous products in the blood of uremic animals.

4. If cross transfusion is started within 90 minutes after intravenous administration of the MLD100 of mercuric chloride, the test animals remain clinically well and their blood creatinine remains normal or nearly normal.

5. If cross transfusion is started within 180 minutes after intravenous injection of the MLD100 of mercuric chloride. the test animals become clinically ill and the blood creatinine rises, but the animals eventually recover.

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Effect of Celiac Ganglionectomy and Thoracolumbar Sympathectomy or Adrenal Medullectomy on Histamine Ulcer in Dogs. (17796)

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In former reports from these laboratories it has been shown that postganglionic sympathectomy of the upper gastrointestinal tract through excision of the celiac or of the celiac and superior mesenteric ganglia accelerates the production histamine provoked peptic ulcer in dogs(1), and that preganglionic sympathectomy by excision of the thoracolumbar sympathetic trunks does not alter the sensitivity of dogs to histamine ulcer significantly from the normal(2). The present studies have been undertaken to elaborate upon these observations and to clarify if possible the mechanism of accelerated ulcer production following celiac ganglionectomy.

Experiments. The healthy, dewormed, adult, mongrel dogs used in these experiments were divided into 4 groups as follows: Group I, dogs in which the celiac ganglia was removed and both thoracolumbar sympathetic chains were excised. Group II, dogs in which the medulla of the left adrenal was removed and an adrenalectomy was done on the right side. Group III, dogs with the celiac ganglia removed in addition to a left adrenal medullectomy and right adrenalectomy. Group IV was a control series of normal dogs. All of the dogs in these 4 groups were given injections of histamine-base-in-beeswax as a stimulus to ulcer production after a recovery period of at least 30 days following surgical procedures. The histamine-in-beeswax was prepared according to the method of Code and Varco(3) and injected intramuscularly daily in the afternoon in 30 mg doses, after which time the food pans were removed until the following morning. Each new lot of histamine was tested for potency on a Heidenhain pouch preparation.

Operative Procedures. Celiac ganglionectomy was done by carefully stripping the celiac axis of all adjacent tissue from the aorta well out onto its 3 branches, removing both semilunar ganglia and their branches in one segment. Thoracolumbar sympathectomy was done bilaterally in 2 stages. The 12th and 13th ribs were resected and the sympathetic trunk was removed transthoracically and retroperitoneally from the 8th thoracic ganglia through the 2nd lumbar ganglia along with the splanchnic nerves as far as the celiac ganglia. Adrenal medullectomy was done by incising the gland lengthwise with a sharp knife and curetting the medulla while a rubber shod clamp occluded the blood supply, according to the technic of Houssay and Lewis(4). The right adrenal gland was subsequently removed completely after recovery from the first operation. Animals with a celiac ganglionectomy in addition to an adrenal medullectomy had both procedures done at the same time.

Results. The results are summarized in Table I. I. *Celiac ganglionectomy with thoracolumbar sympathectomy.* Of 12 dogs with celiac ganglionectomy alone, reported previously(1), all developed gastric or duodenal ulcer with 5 or less daily injections of histamine-in-beeswax, however when bilateral thoracolumbar sympathectomy was added as in the present study not one of 13 dogs developed ulcers with 5 daily histamine injections. One of 2 dogs that received histamine during longer periods developed a duodenal ulcer, suggesting that no protection is offered by this type of denervation but rather that thoracolumbar sympathectomy acts in some manner to abolish the accelerating effect on the production of histamine ulcers exerted by celiac ganglionectomy alone.

II. *Adrenal medullectomy.* Bilateral removal of the adrenal medulla in Group II re-

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1. Lillehei, C. W., and Wangensteen, O. H., Proc. Soc. Exp. Biol. and Med., 1948, v68, 369.
2. Lillehei, C. W., and Wangensteen, O. H., unpublished.
3. Code, C. F., and Varco, R. L., Proc. Soc. Exp. Biol. and Med., 1940, v44, 475.
4. Houssay, B. A., and Lewis, J. T., *Am. J. Physiol.*, 1923, v64, 512.

TABLE I.
Ulcer Production in Dogs.
(30 mg histamine-base-in-beeswax injected intramuscularly daily).

Group	Type of procedure	No. dogs	No. inj.	Erosions only*		Ulcers	
				No.	%	No.	%
I	Celiac ganglionectomy + bilateral thoracolumbar sympathectomy						
	a. Short course of histamine	13	5	2	15	0	0
	b. Long " " "	2	10-45	0	0	1	50
II	Adrenal medullectomy	8	5	2	25	3†	37
III	Celiac ganglionectomy + adrenal medullectomy						
	a. Short course of histamine	9	5	3	33	1	11
	b. Long " " "	2	7-44	0	0	2†	100
IV	Normal dogs	16	5	2	12	4	25

* An erosion is a circumscribed destruction of the mucous membrane limited by the muscularis mucosa while ulcers penetrate deeper into the wall.

† 1 perforated ulcer.

sulted in an incidence of ulcer after 5 daily injections of histamine not significantly different from that found in the normal controls. This is similar to the results described previously(2) in a series of dogs with bilateral thoracolumbar sympathectomy.

III. *Celiac ganglionectomy with bilateral adrenal medullectomy.* Bilateral adrenal medullectomy seems to have a similar effect to that of bilateral thoracolumbar sympathectomy in that it also reduces the ulcer abetting influence of a celiac ganglionectomy alone. Two animals given longer courses of histamine both developed ulcers, showing that no protection against histamine ulcers is afforded by this operation.

Discussion. Stomach emptying time which is decreased by celiac ganglionectomy is also decreased by thoracolumbar sympathectomy or by adrenal medullectomy(5). Combining these operations does not alter the stomach emptying time; it remains faster than normal. We have data on total pouch dogs which indicates that neither celiac ganglionectomy nor thoracolumbar sympathectomy changes the gastric secretory response to histamine from that observed before these nerves were cut(5). It therefore seems improbable that changes in the stomach emptying time or gastric acid secretory response to histamine could be the main effect produced by thoracolumbar sympathectomy or adrenal medullectomy when added to celiac ganglionectomy. However it might be suggested as an hypothesis that adrenalin sensitivity in the stomach and duodenal blood vessels of the animals with a postganglionic denervation(6), that is a celiac ganglionectomy, is a factor in the accelerated rate of histamine ulcer production noted. There is a greatly decreased output of adrenalin with either adrenal medullectomy or adrenal denervation by thoracolumbar sympathectomy(7). Anatomical dissection shows that bilateral thoracolumbar sympathectomy effects a denervation of the adrenal glands not obtained by celiac ganglionectomy alone. These operative procedures, which reduce the adrenalin output, when added to a celiac ganglionectomy would then permit less adrenalin stimulation of the sensitized gastric and duodenal vessels. In a similar manner, as noted by Cannon, Lewis, and Britton(8), the denervated cat heart, which is sensitive to adrenalin, responds to excitement with an in-

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5. Lewis, F. J., and Wangensteen, O. H., unpublished data.

6. Hampel, C. W., *Am. J. Physiol.*, 1935, v111, 611.

7. Stewart, G. N., and Rogoff, J. M., *Am. J. Physiol.*, 1919, v48, 397.

8. Cannon, W. B., Lewis, J. T., and Britton, S. W., *Am. J. Physiol.*, 1926, v77, 326.

creased rate until the adrenals and liver have been denervated. It has been shown that adrenalin-in-beeswax increases the susceptibility of normal dogs to histamine provoked ulcer(9). The adrenalin output normally stimulated by histamine administration(10) might then, in the animals with postganglionic denervation and consequent increased sensitivity to adrenalin, be a factor in explaining their increased histamine ulcer susceptibility. This increased histamine ulcer sensitivity is abolished by operations which markedly reduce the output of adrenalin.

None of this work suggests that any of the operations described afford protection against histamine provoked ulcers. Animals given histamine for longer periods up to 45 days developed ulcers just as control animals do.

Conclusion. The potentiating effect of postganglionic sympathectomy, by a celiac ganglionectomy, on histamine induced ulcer in dogs is abolished by a bilateral thoracolumbar sympathectomy or adrenal medullectomy.

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Received March 2, 1950. P.S.E.B.M., 1950, v74.

Nutrition of Animal Cells in Tissue Culture. II. Use of Tweens in Synthetic Feeding Mixtures.[*][†] (17797)

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A previous communication(1) from this laboratory reported the development of a synthetic feeding mixture, No. 199, that maintained chick embryo mesenchyme cells in a living, healthy state for an average period of 4 to 5 weeks, and kept certain cultures alive for over 70 days. During the course of this work, it was considered desirable to supply the cells with a source of water-soluble fatty acid and to devise a means of dissolving certain fat-soluble compounds in a vehicle other than alcohol. Consequently, an extensive investigation was made of the Tween series of surface active agents,[‡] which have been widely used in bacteriological media(2). The results of this study are contained in the present report.

Materials and method. All cultures were prepared from the leg muscle of 11-day-old chick embryos; and the cells were grown di-

TABLE I.
Main Distinguishing Features of Basal Synthetic Feeding Mixtures Employed.

Mixture No.	Composition
24B	Amino acids and vitamins as in Mixt. 199(1), but stock solutions of Vit. A and D in 95% ethyl alcohol. Total alcohol in mixt., 0.1%.
199	Amino acids, vitamins, purines, pyrimidines, intermediary metabolites, and other growth factors. Stock solutions of Vit. A and D and cholesterol dissolved in Tween 80 with min. alcohol. Final conc.: .002% Tween and .01% ethyl alcohol.
281	Same as 199, but stock solutions of Vit. A and D and cholesterol prepared in 95% ethyl alcohol (without Tween). Total alcohol, 0.3%.
313	Same as 199, but with ethyl alcohol brought to the level (0.3%) used in Mixt. 281.

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[*] This investigation was supported, in part, by grants from the National Cancer Institute of Canada and the Ontario Cancer Treatment and Research Foundation.

[†] Grateful acknowledgment is made to Mrs. M. G. Pearce, Mrs. C. J. Porter and Miss O. E. Rockett for technical assistance.

1. Morgan, J. F., Morton, H. J., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.

[‡] Produced by the Atlas Powder Company, Wilmington, Del.

2. Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, v83, 409.

TABLE II.
Average Survival Time in Days of Cultures Tested in Synthetic Mixture 281 Containing 0.3% Ethyl Alcohol and Various Levels of Tweens and Oleic Acid. (Compare Table III).*†

	Final conc. (%)				
Substance tested	.5	.05	.005	.0005	.00005
Tween 20	2.0	4.5	27.5	27.6	26.6
,, 40	2.5	5.3	13.1	15.3	17.5
,, 60	2.8	4.0	16.8	11.5	16.0
,, 80	2.8	4.5	15.5	14.5	17.0
,, 85	2.0	5.8	27.3	16.6	22.0
Oleic ac.	2.0	5.2	21.5	17.0	32.0

* Each value represents average survival time of at least 6 cultures.

† Average life of control cultures in Mixture 281 was 26.2 days, and in Mixture 199 was 33.3 days.

rectly on the surface of the glass of standard pyrex test tubes by the roller-tube technic(3). The preparation of synthetic Mixture 199, and its basic stock solutions, has already been described(1). It should be mentioned that Mixture 199 contains 0.01% ethyl alcohol. This minimal amount of alcohol was found necessary in order to dissolve vitamin A, vitamin D and cholesterol before bringing them into final solution in the Tweens. In preliminary experiments the effect of the Tweens was studied in a simple synthetic mixture (24B). The majority of the experiments, however, were carried out either in Mixture 281 or in Mixture 313, which are modifications of Mixture 199(1). The main distinguishing features of these various mixtures are indicated in Table I. It should be emphasized that all solutions tested were made up in modified Tyrode's solution(4).

Tweens 20 (monolaurate), 40 (monopalmitate), 60 (monostearate), 80 (monooleate) and 85 (trioleate) were prepared as 5.0% stock solutions in glass-distilled water and were further diluted to appropriate levels in glass-distilled water. Oleic acid (redistilled) was dissolved in 95% ethyl alcohol to a concentration of 5.0% and was subsequently diluted in water immediately before use. All solutions were sterilized by passage through UF fritted glass filters (Corning). Full details of the culture assay procedures were described previously(1). Under the conditions of the experiments, only differences in average survival time greater than several days were considered significant.

Experimental. Preliminary studies were carried out in which Tweens 20, 40, 60, 80 and 85, at final concentrations ranging from 0.5% to 0.00005%, were tested in a relatively simple synthetic mixture (No.24B, Table I). These initial experiments indicated a definite toxicity at 0.5% and 0.05%, whereas concentrations of 0.005% or less were not inhibitory. Accordingly, a low concentration (0.002%) of Tween 80 was incorporated in all subsequent mixtures(1). With the development of Mixture 199, which was more complete than Mixture 24B, it became possible to study the action of the Tweens under more favorable conditions, and the preliminary experiments based on Mixture 24B were repeated with Mixture 281. The results are shown in Table II.

As in the earlier experiments, it was found that Tween concentrations of 0.5% and 0.05% were decidedly toxic for the cells (Fig. 1 and 2) whereas lower levels were not. It was observed that in low concentrations of Tween 20 the cells remained alive for much longer periods than in other members of the Tween series. This longer survival was not confirmed, however, in later experiments. Free oleic acid was also tested at several levels in order to determine whether the toxic effect of high concentrations of Tweens 80 and 85 could be attributed to their content of unesterified fatty acid(5). Because only slight toxicity was observed with concentrations of oleic

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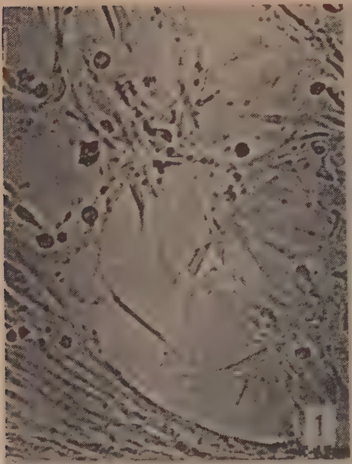
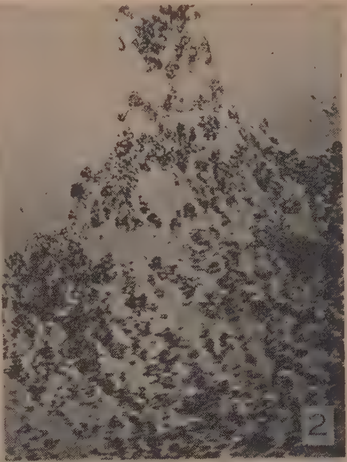
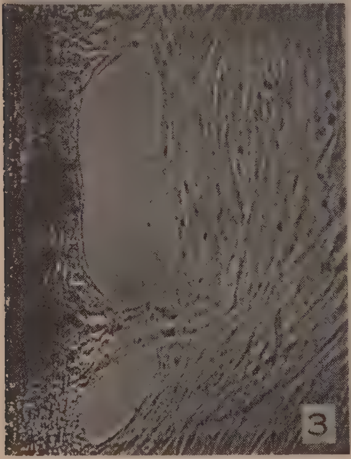
3. Gey, G. O., *Am. J. Cancer*, 1933, v17, 752.
4. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.
5. Davis, B. D., *Arch. Biochem.*, 1947, v15, 351.







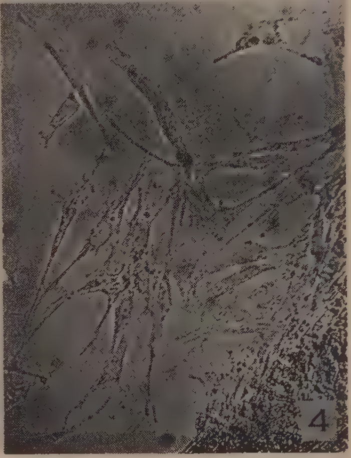


FIG. 1. Living cells from a healthy roller-tube culture of chick embryo mesenchyme tissue after 3 days in synthetic mixture No. 281 (See Table I). × 120.

FIG. 2. Cells from a degenerating culture from same series after 3 days in synthetic mixture No. 281 plus 0.05% Tween 60. (Compare Fig. 1). × 120.

FIG. 3. Living cells from a healthy roller-tube culture of chick mesenchyme tissue after 32 days in synthetic mixture No. 199 (containing 0.002% Tween 80). × 120.

FIG. 4. Living cells from a healthy culture of chick embryo mesenchyme tissue after 31 days in synthetic mixture No. 199 with a higher level of Tween 80 (0.005%). × 120.

acid far in excess of those that could conceivably be present in the Tween solutions, even after storage(6), the commercial preparations were employed without further purification. The beneficial effect of 0.00005% oleic acid, shown in Table II, was not obtained in previous or subsequent experiments.

It was observed that cultures maintained in Mixture 281 plus low concentrations of Tween survived for much shorter periods than those in Mixture 281 alone or in Mixture 199 (Table II). These findings suggested that even low concentrations of Tweens appear

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TABLE III.
Average Survival Time in Days of Cultures Tested in Synthetic Feeding Mixtures Containing Tweens at 0.0005% and Ethyl Alcohol at 2 Levels.*

Compound tested	.3% alcohol	.01% alcohol
Tween 20	27.6	28.6
,, 40	15.3	36.0
,, 60	11.5	28.5
,, 80	14.5	37.2
,, 85	16.6	33.5
Controls	25.4†	33.3‡

* Each value represents average survival time of at least 12 cultures.
† Mixture 281.
‡ Mixture 199.

TABLE IV.
Effect of Various Concentrations of Tween 80 upon the Survival Time of Cultures Maintained in a Synthetic Feeding Mixture Containing 0.01% Ethyl Alcohol.*

Tween 80, %	Survival, days
0.5	2.2
0.05	7.0
0.005	31.2
0.002†	33.3
0.0005	40.5
0.00005	32.7

* Each value represents average survival time of at least 6 cultures.
† Mixture 199.

somewhat toxic when incorporated in media containing a relatively small amount of ethyl alcohol (0.3%). It was decided, therefore, to attempt to avoid injurious levels of alcohol by dissolving the alcoholic concentrates of vitamins A and D and cholesterol, at their appropriate dilutions, directly in the levels of Tween to be tested. Table III shows the survival time of cultures in the 5 Tweens at a level of 0.0005% and in 2 levels of alcohol. It is apparent from the results of these experiments that a reduction in the alcohol content of the medium eliminates the toxic effect associated with the presence of low concentrations of Tweens. It is also interesting to note that the presence of alcohol does not appear to influence the survival time of cultures maintained in media containing a low concentration of Tween 20. In this respect, Tween 20 would seem to differ from the other Tweens that were tested.

For studies on cell nutrition, the addition of an unsaturated fatty acid to the synthetic mixtures was considered preferable to the addition of a saturated one. From the Tweens available, therefore, Tween 80, a monooleate, and Tween 85, a trioleate, were selected. It was found, however, that solutions of Tween 85 rendered the mixture so turbid that it was difficult to observe the cells, and for this reason all subsequent studies were confined to Tween 80. The effect of various concentrations of this Tween, tested in Mixture 199 containing a minimal amount of alcohol (0.01%), is shown in Table IV. These results confirmed the toxic levels of Tween 80 already established and indicated that concentrations from 0.005% to 0.00005% are suitable for studies in animal cell nutrition (Fig. 3 and 4).

Discussion. The toxicity of lipids for animal cells in tissue culture has long been assumed. Baker and Carrel(7) found that crude lipids of serum, chicken brain, chicken liver, egg and embryo tissues exerted an inhibitory action on the growth of fibroblasts; and Mayer(8) observed a toxic effect with lipids from brain tissue. Margoliash(9) found that both adult and embryo tissue extracts, used as growth stimulants for tissue cultures, could be considerably improved by extraction with fat solvents. Carminati(10), on the other hand, observed that a synthetic distearyl lecithin had a distinct growth-promoting effect on chick heart cultures. Also, Davidson and Waymouth(11) reported that defatted chick embryo extract is less effective than whole extract in promoting growth and that cultures deteriorate more quickly in defatted plasma than in whole plasma. It would seem, then, that a detailed study of the effect of individual lipid fractions is necessary before the relationship of lipids to cell growth and survival can be established with any certainty.

The present experiments have established that all Tweens tested are definitely toxic to animal cells in tissue culture at concentrations

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11. Davidson, J. N., and Waymouth, C., *Biochem. J.*, 1946, v40, 568.

of 0.5% and 0.05%. These results are at some variance with those reported for bacteria. Dubos and Davis(2), who studied the growth of tubercle bacilli in liquid media, found that Tweens 20 and 40 were inhibitory at 0.005% or higher, but that Tween 80 was not inhibitory up to 0.3%. The present experiments have shown no marked differences in the degree of toxicity of different members of the Tween series, since all Tweens tested have been consistently toxic at a concentration of 0.05%. It should be pointed out, however, that no detailed comparative studies on the relative value of the various members of the Tween series have been reported for bacteria.

The present experiments have established that oleic acid and the various Tweens are toxic to animal cells in tissue culture at approximately the same concentrations. The toxic level of oleic acid for tissue cells (0.5% to 0.05%) is somewhat higher than that reported for certain lactic acid bacteria, where levels of 0.004% and 0.001% were found to be inhibitory(12,13). It is also interesting to note that the toxic action of oleic acid for tissue cells is not prevented by the presence of cholesterol, calciferol or α-tocopherol, which are present in Mixture 199. These compounds were found(13) to reverse the inhibitory effect of oleic acid upon lactic acid bacteria.

The results reported in this communication, which are based on approximately 1200 cultures, have not revealed a beneficial effect upon cell growth or survival that can be attributed definitely to Tween alone or to oleic acid. Although these studies have not established whether lipids in general are toxic for tissue cultures, they have shown that certain esterified fatty acids can be used without harmful effects provided suitable concentrations are employed. Since it is possible that animal cells in tissue culture may require a source of fatty acids, the use of non-toxic concentrations of Tween would appear to be desirable in synthetic media studies. The ability of these surface active agents to dissolve fat-soluble vitamins and cholesterol and to hold them in solution in aqueous media can also be utilized as a convenient means of eliminating all but minute traces of alcohol from the synthetic mixtures.

It should be emphasized that the present experiments were carried out in media that were completely synthetic. Also, the use of long-continued homogeneous cell strains was not considered practicable since these are ordinarily cultivated in a plasma coagulum and must be supplied with a nutrient fluid containing chick embryo extract. It was felt that the lipases present in such complex natural media would hydrolyze any Tween added and so confuse the results. For the same reason, Tween toxicity values obtained by rat feeding experiments(14) cannot be related to the present findings.

Summary. The effect of Tweens 20, 40, 60, 80 and 85, and oleic acid, upon the survival of chick embryo mesenchyme cells maintained in a completely synthetic feeding mixture has been studied over the concentration range of 0.5% to 0.00005%. Levels of 0.5% and 0.05% were definitely toxic whereas lower concentrations were not. Relatively small amounts of ethyl alcohol (0.3%) were inhibitory when used together with low concentrations of Tweens. Reduction of the alcohol content to a minimum (0.01%) makes it possible to employ the Tweens as a source of water-soluble fatty acids and also to dissolve fat-soluble compounds in aqueous synthetic feeding mixtures.

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Received March 3, 1950. P.S.E.B.M., 1950, v74.

Expulsion-Crescent: A New Pathological Red Cell Figure in Human Peripheral Blood. (17798)

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From Laboratory for the Study of Human Blood, The Robinson Foundation, New York City.

A new red cell figure was first seen by the author in 1942, in the blood of a Negress, whose possible sickling condition was under investigation. She had a moderate anemia and a greatly enlarged and granulated, apparently cirrhotic liver. The new figure was first mistaken for some type of sickling, but sickling was not found. It is not without significance that in the method of investigation at that time, there was employed a suspension of a droplet of finger-blood in a drop of the Blood-Group-Enzyme (BGE)-broth, both on a slide, and covered with a cover slip. This was the method the author began to use in 1941 and routinely applied since then, for the rapid detection of sickling(1) or, to define it better, for the detection of red cell sensitivity to the BGE, both in Negroes(2) and Caucasians(3).

The figure observed and later designated Expulsion-Crescent, consisted of 2 parts: 1. a highly refractile and crescent-like structure, 2. a smooth and pale, vesicle-like structure; resembling a spherocyte closely fitting into the legs of the crescent. Although the mutual relation between these two parts was at first not understood, it was clearly recognized in a case subsequently seen, which was that of a girl with a pluriglandular disturbance, a member of a family in which 5 other members were reported as having died of Wilson's disease (lenticular degeneration), a form of cirrhosis of the liver. Fig. 1 is the structure here described found in this patient, by the method indicated.

Continued observation of this structure revealed that it is not a static one, but that it is in motion. The spherical structure is not a spherocyte simply lying, by chance, between the legs of the crescent. Both crescent and vesicle are 2 integral parts of the same single cell and it will be indicated that they represent apparently different materials with different qualities, and different functions with different end results. Numerous observations, constantly duplicating the picture of Fig. 1, have proven that. In this picture, the development of an Expulsion-Crescent was observed for about 10 minutes.

We see a dense refractile material take shape of a crescent, seemingly retract and expel another substance which is smooth, spherical in shape, and is swelling, paling and vanishing, finally a cell of apparently reduced size remaining. With slight insignificant differences, it is always the same event, as seen in the collection of Expulsion-Crescents in Fig. 2.

In this Fig. 2, various Expulsion-Crescents are shown as observed during the period of separation of the 2 different cell components described above, and after that, when the separation is completed and the crescent remains. There is a striking similarity among all figures, revealing their identity, thus precluding their adventitious nature.

A study of Expulsion-Crescents in 17 cases, from 1945 to 1948 inclusive, established definitely:

1. that this structure of the Expulsion-Crescent, if sought for under proper conditions, is not rare,

2. that it is commonly associated with parenchymatous liver disease, especially cirrhosis,

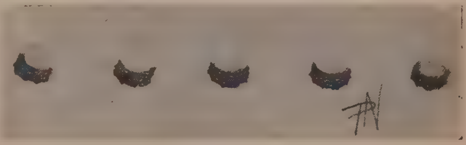

FIG. 1.
Expulsion of the smooth, swelling and dissolving vesicle by the retracting, refractile, crescentic part of the red cell in 5 stages. Observation for about 10 minutes, of washed red cells suspended in a blood-group-enzyme broth.

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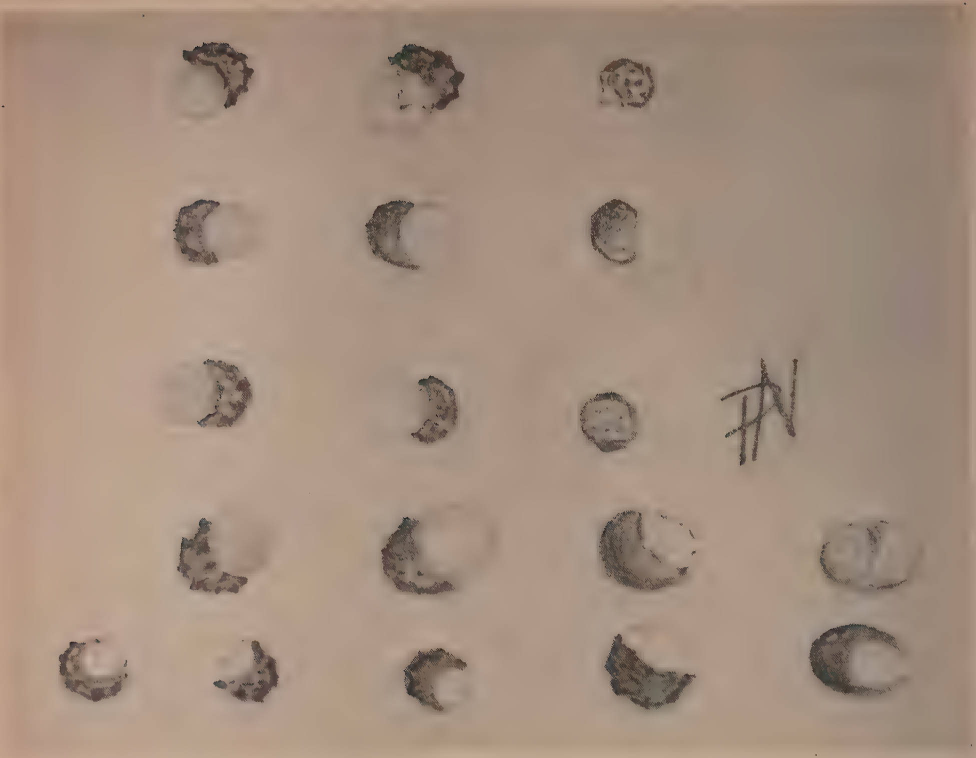

FIG. 2.
Various typical expulsion-crescents in motion (first 4 lines), and resting after the expulsion (fifth line), in a case of cirrhosis of the liver (A.Spr.).

3. that it is, apparently, the expression of a peculiar blood-destructive process,

4. that this blood-destructive process may in the main be not unlike the blood-destructive process acting in the sickling phenomenon, *i.e.* that this new crescent-like structure may belong to the category which the author has begun to designate in 1945 "hemolysis of sickle cell type".

This latter view is corroborated by the fact that in some cases Expulsion-Crescents are seen simultaneously with "block-partition" figures, the blood-destructive analogue in Caucasians and non-sickling Negroes(3), of the sickling condition in sickling Negroes. The following Fig. 3, depicts this concurrence of Expulsion-Crescent and block-partition in another case of cirrhosis of the liver.

Cirrhosis of the liver seems to be the prototype disease for the appearance of Expulsion-Crescents. In the 17 cases, in which such characteristic crescents were observed, actual liver disease (cirrhosis) was found in 6 cases or in 35.4%. Liver involvement evidenced by an increased icteric index of the plasma was found in three more cases, raising the percentage of liver involvements to 53.1%. The diagnoses of the remaining cases testify to the seriousness and significance of the disease conditions in which this type of blood destruction may be found: malignancies, toxemias of pregnancy, hemolytic anemias.

As to the nature of this phenomenon, it is highly suggestive to regard the separation of the two substances, the one refractile and dense, the other one smooth and dissolving, in the light of separation of two similar substances in the sickling cell of Negroes, under BGE influence. In the 4 regular stages observed in the sickle cell development, the sec-

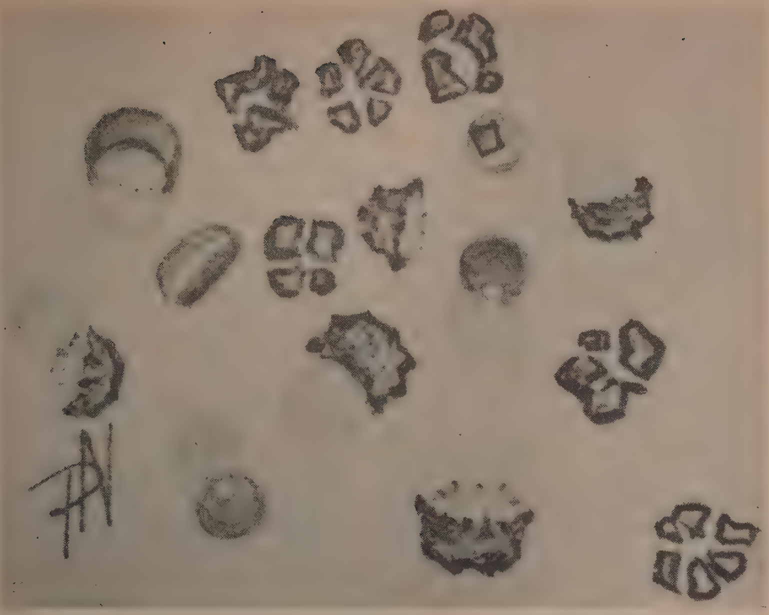

FIG. 3.
Expulsion-crescents and block-partition figures together in a case of cirrhosis of the liver (X.H.). Spontaneous appearance in oxalated blood after 36 hours.

ond stage would correspond to the Expulsion-Crescent. The following Fig. 4 is taken from a previous paper(3). It illustrates the sickle cell development under BGE influence, with the second stage in principle strikingly similar to the Expulsion-Crescent.

Inasmuch as this crescent is achromatic, it would seem to lack the properties of nuclear material. This opinion is reinforced by the fact that the expelled portion quickly dissolves.

Regarding the method of demonstration of the Expulsion-Crescent, later experience has revealed that the application of the BGE is not an absolute necessity. Like the sickling phenomenon, which is accelerated by the BGE but appears also spontaneously, either in washed red cells or in the oxalated blood, the Expulsion-Crescent can thus be found without the application of the BGE, both in washed red cells and in the oxalated blood. The crescentic structures of the Fig. 3 had appeared in the oxalated blood after a lapse of time of about 36 hours. The method commonly used by me at present, is the microscopic observation on a slide covered with a cover slip, of red cells washed three times in saline. Characteristic changes in the erythrocytes will appear within about 30 minutes.

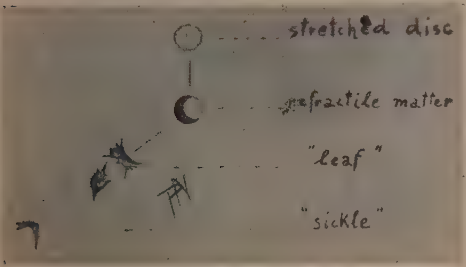

FIG. 4.
The 4 regular stages in the sickle cell development under BGE influence.

Summary. A new pathological red cell figure is described, apparently presenting another blood-destructive process of the "sickle cell type", and occurring in Negroes and Caucasians alike, in both of which cirrhosis of the liver is the probable prototype disease.

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Accelerator-Globulin and Prothrombin: Preparation of Accelerator-globulin Free Plasma. (17799)

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In recent years, considerable clarification of the conversion phase of the blood clotting mechanism has resulted from the introduction of the concept of a "convertibility" factor (1-3), and more recently, from the conclusive demonstration of the existence of an accessory clotting factor which accelerates the conversion of prothrombin to thrombin(4-7). Many discrepancies which have been observed in different species, under slightly modified experimental conditions, and with different methods of quantitative prothrombin estimation, have been reconciled, in part, by an understanding of the role of the accelerator factor in the coagulation of blood.

The present investigation was undertaken to elucidate further the qualitative and quantitative nature of the accelerator factor. Plasma was subjected to the action of chemical, physical, and enzymatic agents. With such agents, denaturation, adsorption, precipitation, and inhibition phenomena were studied and evaluated with respect to the activity of the accelerator factor. During the course of our studies, a method was found by which accelerator-globulin could be removed from bovine plasma without concomitant removal of the prothrombin. This has provided an accelerator-globulin-free solution of plasma prothrombin which has proved very useful as a reagent for studies of the clotting mechanism.

Methods. Throughout this work, the method of Ware and Seegers(8) for the assay of accelerator-globulin activity was used to check our results.* The careful studies of Flynn and Standley(9) have led them to conclude that all of the "accessory factors" described manifest virtually the same effect, and are probably one and the same. However, since we have used the techniques of Ware and Seegers and compared our results only with theirs, we feel justified in this paper in referring to the accelerator factor as Ac-globulin. Prothrombin concentrations were determined by the modified two-stage technic (10-11). Purified thromboplastin, prepared after the method of Chargaff *et al.*(12), slightly modified by Layton,† and centrifuged at 5000 r.p.m. to remove the particulate material, was used. This product is stable, gives consistent, reproducible results, and does not manifest Ac-globulin activity. Armour bovine fibrinogen was used exclusively. Our data indicate that it is free of Ac-globulin.

Results. The data obtained when oxalated bovine plasma was subjected to the action of agents which involved basic chemical, physi-

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* We are indebted to these investigators for the generous supply of their purified prothrombin.

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† Personal communication.

cal, and enzymatic reactions, showed great variations in the response of Ac-globulin and prothrombin to such agents. Agents used included ultraviolet light; buffer solutions over a wide range of pH values; denaturing reagents such as urea, alcohol, detergents and phenol; adsorbents such as salts of barium and phosphorus, and silicon compounds; a variety of salts of heavy metals; and proteolytic enzymes such as trypsin, pepsin, and papain. Variable degrees of destruction occurred, and with most of the agents, a decrease in Ac-globulin activity did not occur without comparable loss of prothrombin. With some agents, the prothrombin, although only moderately reduced quantitatively, was so altered qualitatively that its reactivity with respect to the conversion phase of clotting was unpredictable. A few agents were effective in reducing prothrombin concentration without greatly altering Ac-globulin activity. During the course of these investigations, it was recognized that a plasma, containing prothrombin, but free from Ac-globulin would be very useful in the study of certain components of the clotting system, and particularly, as a source of prothrombin in the assay of Ac-globulin. None of the agents tested were sufficiently selective to quantitatively remove either Ac-globulin or prothrombin without effecting some concomitant loss of the other.

Method. Because of the complexity of the variables in the clotting system, the method we have used for preparing Ac-globulin free plasma is a compromise and a balance of many factors, and does entail some loss of prothrombin.

1. To each 100 ml of oxalated beef plasma, add 17 g of kaolin (NF powder); mix thoroughly, and immediately place kaolin-plasma at –20°C to –60°C; swirl every few minutes until completely solidified.
2. Store kaolin-plasma at –20°C to –60°C for 24-48 hours.
3. Thaw out the kaolin plasma at room temperature, swirl once or twice, and centrifuge for 15 minutes at 5000 r.p.m. at 5°C.
4. Decant the plasma, and to it add slowly, with constant stirring, an equal volume of 1.5% $HgCl_2$ solution. Do not centrifuge.
5. To 100 ml of the $HgCl_2$-plasma add immediately 20 g of Bacto-Egg Albumin, Coagulated.[‡] The egg albumin is first moistened by the addition of water, followed by centrifugation and decantation.
6. Stir mixture gently for 5 minutes.
7. Centrifuge the mixture for 30 minutes at 5000 r.p.m. at 5°C.
8. Decant supernatant; again add moistened coagulated egg albumin in a concentration of 20 g per 100 ml, and stir as before.
9. Centrifuge as in step 7. Decant and filter (filter paper) supernatant.
10. To approximately 1 ml of the final product, add a small crystal of $SnCl_2$. If a white milky precipitate results, the mercuric ions have been removed, if the precipitate is black, mercuric ions are present, and further treatment with coagulated egg albumin is necessary.
11. Put 1.2-1.3 ml amounts of $HgCl_2$-plasma in separate capillary tubes, and store at –20°C to –60°C. Use a fresh tube for each Ac-globulin determination. Do not use until the $HgCl_2$-plasma has been frozen for at least 1 hour.
12. Fibrinogen, small amounts of which may be present in the final preparation, precipitates on thawing, and may be removed readily.

The product, which henceforth we shall refer to as $HgCl_2$-plasma, has a prothrombin concentration of 50-60 units per ml depending upon the initial prothrombin concentration of the native beef plasma. The $HgCl_2$-plasma has a pH 7.2, no specific buffer having been added. The amount of prothrombin adsorbed by the kaolin is fairly constant, being approximately 46%. The prothrombin concentration of the kaolin treated plasma again is reduced by one-half because of the dilution effected by the addition of an equal volume of 1.5% $HgCl_2$ solution. The $HgCl_2$ precipitates an additional 2-3% prothrombin. Therefore the prothrombin concentration of the $HgCl_2$-plasma is approximately one-fourth that of the original beef plasma. Although the quantitative yield of prothrombin is only approximately one-half qualitatively it apparently is unaltered by the procedure. The

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[‡] Difco Laboratories, Detroit, Mich.

$HgCl_2$-plasma does not manifest Ac-globulin activity, and the antithrombin titer is extremely low. The prothrombin concentration of the $HgCl_2$-plasma when the latter is stored at –20°C to –60°C for 4 months remains unaltered.

In all prothrombin determinations of the $HgCl_2$-plasma, the modified two-stage method of assay was used(11). However, because no Ac-globulin is present in the $HgCl_2$-plasma, it is necessary to add a larger amount of beef serum (Ac-globulin) to the incubation mixture than is normally used. We have found that a 1-400 total dilution of beef serum insures complete conversion, but even with this concentration, the conversion rate is slower than it is in normal plasma.

The method of assay of Ac-globulin, as well as studies on the kinetics of the conversion phase of the clotting system, will be presented in detail in a subsequent paper. A modification of the 2-stage procedure(8) has been devised. It is sufficient to present here only the tenets on which the quantitative estimation is based. The amount of Ac-globulin in any given plasma is manifested as a function of 3 variables: (1) the time when prothrombin conversion begins, (2) the rate of prothrombin conversion, and (3) the yield of thrombin. Therefore a quantitative measure of Ac-globulin can be represented by an area which is circumscribed by the activation curve (clotting time plotted against incubation time) and by straight lines through a constant, arbitrarily selected point on each axis. This area can be determined by the square counting method, one of the quadrature methods, or more simply with a planimeter. The amount of Ac-globulin of an unknown is determined by comparing the area of the unknown with that of a normal control. By referring the area to a standard reference curve, the Ac-globulin activity can be expressed in percent of normal. The prothrombin concentration is adjusted so that only small quantities of plasma are required for the measurement of Ac-globulin, thereby greatly minimizing the effect of antithrombin on the conversion rate and thrombin yield. A concerted effort was made to devise a one-stage technic for the quantitative determination of Ac-globulin. The results of such an attempt were unsatisfactory because the three variables, noted above, could not be isolated, and no one variable could be interpreted as being specifically related to Ac-globulin activity.

Summary. The chemical and/or physical relationship between prothrombin and Ac-globulin apparently is very close as evidenced by the inability to denature, adsorb, precipitate or otherwise inactivate completely, and at times partially, the one, without completely or partially removing or inactivating the other. A method is described whereby oxalated bovine plasma may be rendered free from Ac-globulin, but not from prothrombin.

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Relation of Oxygen to Photoreactivation of Bacteria After Ultraviolet Radiation.* (17800)

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The loss of capacity for cell division resulting from exposure to ultraviolet radiation has been shown in some instances to be restored by subsequent illumination with visible light

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(1-5). Since the mechanism of this "photoreactivation" remains obscure, it has seemed important to test the possibility that photodynamic action, a process requiring molecular oxygen(6), is involved. The data reported herein show that O_2 is not essential, thus eliminating this possibility.

Methods. Washed cell suspensions in 0.5% NaCl solution buffered with 0.1 M KH_2PO_4 - Na_2HPO_4, usually containing about 1,000 cells per ml, were exposed to radiation from an intermediate pressure mercury arc through a Coret D filter. The intensity was approximately 1 x 10^4 ergs/cm^2/sec. of wavelengths 0.27 μ to 0.313 μ; the method of dosage is described by Blum and Price(7). In the sea urchin's egg, complete recovery of rate of cell division occurs after delay by such dosage (4,7). For photoreactivation suspensions were illuminated in Thunberg tubes placed at a distance of 12 inches from a bank of four 20 watt 'fluorescent' lamps ('Daylight' type). The reactivation is brought about by wavelengths in the blue, violet and near ultraviolet(1-4), in which the fluorescent lamps are relatively rich. Aliquot portions of the irradiated cell suspension were transferred to a series of Thunberg tubes arranged in pairs. One tube of each pair was wrapped in black cloth and placed adjacent to the corresponding illuminated tube to serve as a 'dark control'. Tubes were withdrawn a pair at a time at intervals during illumination and plate counts made from each tube. The influence of oxygen was determined in similar manner except that of each pair of tubes one was evacuated. Both then were illuminated and plate counts made. The virtually complete removal of oxygen from the evacuated tubes was indicated by the extinction of luminescence in a dilute suspension of *Photobacterium phosphoreum* connected to the same vacuum line. Bacterial luminescence is a particularly sensitive indicator for the presence of molecular oxygen(8).

Results. The photoreactivation of the facultative anaerobe, *Escherichia coli,* strain B, is illustrated in Fig. 1 by a typical experiment. Reactivation was not found to be very sensitive to temperature in the range of 22° to 37°C, and was about the same whether the hydrogen ion concentration was acid or near neutrality.

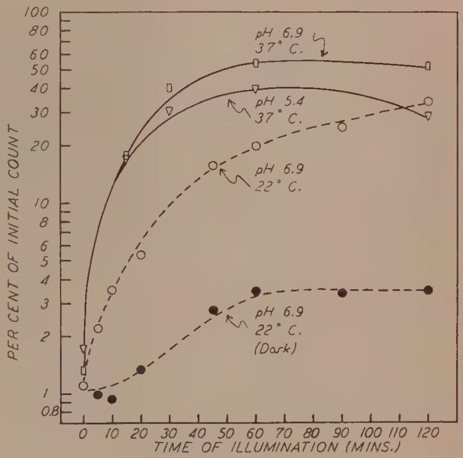

FIG. 1.

Percent of initial count of a suspension of *E. coli* after exposure to ultraviolet radiation ($\sim$ 2.5 $\times$ 10^6 ergs/cm^2; λ = 0.270 μ to 0.313 μ), and subsequent illumination with visible light† for various lengths of time (open symbols, upper 3 curves) and in the dark (solid disks, lowest curve). Broken and solid lines refer to 2 different cell suspensions, each containing 880 cells per ml before exposure to ultraviolet radiation, the former illuminated at 22°C and the latter at 37°C. The pH of the salt solution was 6.98 in all except that represented by the next to uppermost curve (triangles) which was 5.4.

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† Zero time refers to the time at which illumination with visible light was begun. A few minutes elapsed between the end of ultraviolet irradiation and the beginning of illumination, during which all specimens were kept in the dark.

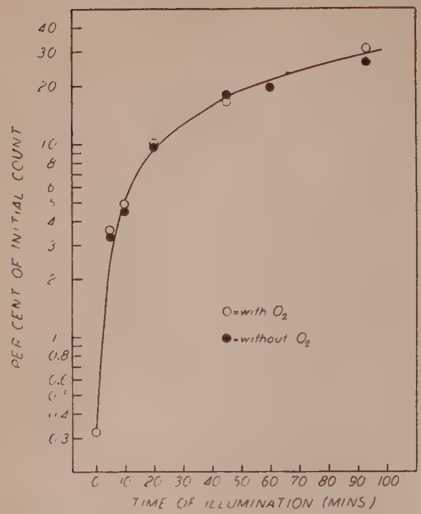

FIG. 2.

Photoreactivation† of *E. coli* in presence and absence of O_2, after exposure to ultraviolet radiation (2.5×10^6 ergs/cm²; $\lambda = 0.270\ \mu$ to $0.313\ \mu$). Initial plate count before irradiation was 1,100 cells per ml; pH of the salt solution was 6.9; temperature during illumination, 22°C.

That photoreactivation takes place in the virtual absence of molecular oxygen is demonstrated by the data in Fig. 2. The close agreement of the counts indicates that photoreactivation is completely independent of O_2. Dulbecco[3] has recently observed that photoreactivation of bacteriophage, in the presence of the host cells, is also independent of molecular oxygen, supporting the conclusion that photodynamic action is not the mechanism involved.

Efforts to study the same phenomenon in a more strictly aerobic organism, *Bacillus cereus*, were unsuccessful because no appreciable photoreactivation could be demonstrated. Typical data are illustrated in Table I. In these experiments, suspensions of wholly vegetative cells were obtained by inoculating glucose nutrient agar plates with spores and washing the cells after 5 hours incubation at 37°C. Microscopic observation confirmed that spore germination had been complete, and new sporulation had not begun. It will be noted that considerably higher dosages of ultraviolet were required to reduce the plate count than in the case of *E. coli*, and that subsequent illumination caused no significant increase. Thus although photoreactivation

TABLE I.

Influence of Illumination by Visible Light, on Plate Counts of *Bacillus cereus* After Exposure to Ultraviolet Light. Vegetative cells from 5 hour cultures on 1% glucose, nutrient agar plates at 37°C, washed and resuspended in 0.5% NaCl solution buffered at pH 7 ± .02 by 1 M KH_2PO_4-Na_2HPO_4. Suspension A, irradiated after two days' storage in the refrigerator; B, same suspension irradiated after 6 days; C, different suspension, after 3 weeks' storage in the refrigerator.

Initial No. of cells per ml	Dosage ultraviolet radiation ergs/cm²	Plate count shortly after exposure to ultraviolet radiation	% initial count	Time of standing† (min.)	Illuminated		Dark	
					Plate count	% initial count	Plate count	% initial count
A. 5060	6.25×10^6	505	10.0	5	526	10.4	588	11.6
				10	374	7.4	465	9.2
				40	288	5.7	367	7.3
				80	177	3.5	101	2.0
B. 1490	6.25×10^6	199	13.4	5	9	0.6	44	2.9
				10	69	4.6	126	8.4
				20	8	0.5	30	2.0
				40	10	0.7	14	1.0
C. 180	2.7×10^6	10	5.5	5	8	4.4	*	*
				10	7	3.6	7	3.6
				20	11	6.1	*	*
				30	*	*	10	5.5
				40	11	5.9	*	*
				80	10	5.5	*	*
				95	8	4.4	3	1.7

* Not plated.

occurs in diverse types of cells and is probably a widely distributed phenomenon, it is not always readily demonstrable.

Summary. Molecular oxygen is not essential for the photoreactivation of *E. coli* after inactivation by ultraviolet radiation, demonstrating that photodynamic action is not involved.

Vegetative cells from young cultures of *Bacillus cereus* show no appreciable photoreactivation.

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Comparative Action of Aureomycin, Chloromycetin, Neomycin, Q-19, and Polymyxin B Against Gram Negative Bacilli. (17801)

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Although the number of effective antibiotics continues to increase, infections due to gram negative bacilli still pose many therapeutic problems. In the present study, the sensitivities of strains of *Pseudomonas aeruginosa, Aerobacter aerogenes, Escherichia coli* and *Proteus vulgaris* to neomycin, Q-19, polymyxin B, aureomycin, and chloromycetin have been determined. Streptomycin-resistant variants of *E. coli* have been compared with the resistant variants of the same strain appearing with neomycin and Q-19. Studies on the mode of action of neomycin and Q-19 are also presented.

Materials and Methods:* Neomycin, produced by a member of the genus *Streptomyces* and discovered by Waksman(1,2), has been shown to be active against a wide variety of both gram negative and gram positive organisms. Q-19,† produced by a strain of *Bacillus circulans* and considered to be related to the polymyxin-aerosporin type of antibiotic was discovered by Murray and Tetrault(3). They reported it to be more active against gram negative than gram positive organisms. The activity of preparations from *Bacillus polymyxa* and a related organism, *Bacillus aerosporus,* against gram negative organisms was reported almost simultaneously by 3 groups of workers(5-7). A crude preparation of polymyxin B prepared by the Pfizer laboratory was used in this study. Aureomycin and chloromycetin, whose properties and effective bacterial spectra have been recently reviewed, were used as standards of comparison for the newer antibiotics(8-10).

Neomycin, Q-19, polymyxin B, and aureomycin were stable and readily soluble in concentrated solutions of sterile saline. Fresh stock solutions containing 10 to 25 mg per ml were prepared weekly and appropriate dilutions made on the day of each experiment. Chloromycetin was not as soluble but a stock solution containing 250 μg per ml was made by heating saline solution and slowly adding the drug. All stock solutions were stored in the refrigerator. Twenty strains each of *A. aerogenes, P. aeruginosa, E. coli* and *P. vulgaris* isolated from urines sent for culture to the bacteriology laboratory of the University of Minnesota Hospitals were used as the test organisms. The drug sensitivity of each strain was determined by a broth dilution method. In order to get direct comparisons, simultaneous determinations were made of the sensitivity of each strain to the 5 drugs. One-half ml of a 1:100 dilution of an 18-hour culture of bacteria in tryptose broth was added to tubes (13 x 100 mm) containing 0.5 ml of two-fold serial dilutions of each drug. Since polymyxin B was not available during the initial phases of this study, comparison of its activity with that of the other 4 antibiotics was made on only 11 strains of *E. coli,* 10 strains of *A. aerogenes,* and 10 strains of pseudomonas. The end point of the sensitivity test was determined by selecting the lowest concentration of drug at which no growth was visible after 18 hours of incubation at 37°C. Repeated determinations of the sensitivity of one strain of *E. coli* to neomycin, streptomycin, chloromycetin, aureomycin, and Q-19 revealed a variability of only one tube in the method. While variation in absolute values occurred, the relative sensitivities to the 5 drugs remained almost constant. Those strains whose growth was not inhibited by 31.2 μg per ml of drug were arbitrarily considered to be relatively resistant.

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* Neomycin and Q-19 supplied by Upjohn Co. Aureomycin supplied by Lederle Laboratories. Polymyxin B supplied by Pfizer Corp.

† Q-19 has also been referred to as circulin. It is a different antibiotic than the one described by McLeod(4), which was originally named circulin but has since been renamed polypetin.

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In order to determine whether Q-19 and neomycin induced the penicillin type or the streptomycin type of resistant variant described by Demerec(11), pour plates were prepared of heavy suspensions of *E. coli* in agar containing increasing amounts of streptomycin, neomycin, and Q-19. The strain of *E. coli* used was originally isolated from the urine of a patient. A single colony was seeded on an agar slant which was used as a stock culture for the complete series of experiments. This strain was shown to be most sensitive to Q-19 and equally sensitive to streptomycin and neomycin by the broth dilution method. Large bottles containing agar were inoculated with stock strain of *E. coli* and incubated at 37°C for 18 hours. The organisms were then suspended in saline solution in a concentration of from 1 x 10^{10} to 3 x 10^{10} per ml. One ml of this heavy suspension was placed in Petri dishes and 10 ml of agar containing increasing concentrations of antibiotic was added. After 5 days of incubation at 37° the number of resistant colonies was determined. The results were recorded as the numbers of variants resistant to each concentration of antibiotic per 1 x 10^{10} organisms.

Whether neomycin and Q-19 were relatively bactericidal or bacteriostatic was determined in 2 ways. In the broth dilution method of determining sensitivity, the end point was the first tube showing no turbidity after 18 hours. The presence or absence of micro-organisms in the clear tubes was determined by placing a 0.1 ml aliquot from each of the first 3 clear tubes into 10 ml of tryptose broth. The diluted the drug sufficiently to permit the growth of variable organisms. The rapidity with which Q-19 and neomycin killed organisms was determined by adding 5 ml of a 1:100 dilution of an 18-hour broth culture of the stock strain of *E. coli* to 5 ml of broth containing 20 μg per ml of each of the antibiotics. The number of viable organisms was determined by removing 0.1 ml aliquots immediately, at 40 minutes, 2 hours, 4 hours, 6 hours, and 18 hours, and making colony counts in plain agar plates.

Results of In vitro Sensitivity tests: Escherichia coli—The majority of the strains were sensitive to the 5 antibiotics (Fig. 1). Q-19 was the most effective antibiotic against 13 strains. Five strains were resistant to Q-19, 2 to aureomycin, 1 each to chloromycetin and polymyxin B. None of the 20 strains were resistant to neomycin.

Pseudomonas aeruginosa—Q-19 and poly-

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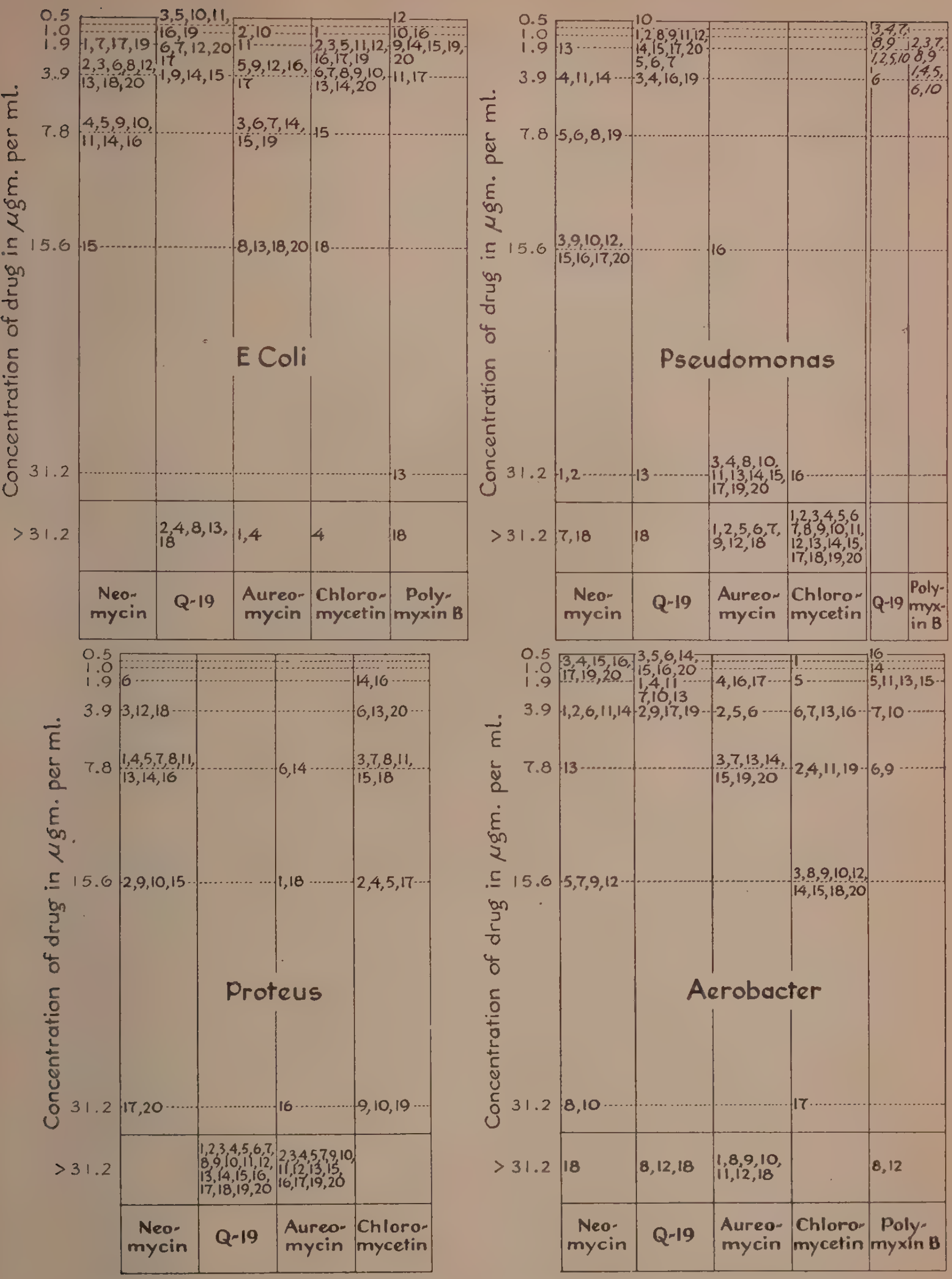

E Coli

Concentration of drug in μgm. per ml.	Neomycin	Q-19	Aureomycin	Chloromycetin	Polymyxin B
0.5		3,5,10,11,			12
1.0		16,19	2,10	1	10,16
1.9	1,7,17,19	6,7,12,20 17	11	2,3,5,11,12, 16,17,19	9,14,15,19, 20
3.9	2,3,6,8,12, 13,18,20	1,9,14,15	5,9,12,16, 17	6,7,8,9,10, 13,14,20	11,17
7.8	4,5,9,10, 11,14,16		3,6,7,14, 15,19	15	
15.6	15		8,13,18,20	18	
31.2					13
>31.2		2,4,8,13, 18	1,4	4	18

Pseudomonas

Concentration of drug in μgm. per ml.	Neomycin	Q-19	Aureomycin	Chloromycetin	Q-19	Polymyxin B
0.5		10			3,4,7,	
1.0		1,2,8,9,11,12,			8,9	
1.9	13	14,15,17,20 5,6,7			1,2,5,10	2,3,7, 8,9
3.9	4,11,14	3,4,16,19			6	1,4,5, 6,10
7.8	5,6,8,19					
15.6	3,9,10,12, 15,16,17,20		16			
31.2	1,2	13	3,4,8,10, 11,13,14,15, 17,19,20	16		
>31.2	7,18	18	1,2,5,6,7, 9,12,18	1,2,3,4,5,6 7,8,9,10,11, 12,13,14,15, 17,18,19,20		

Proteus

Concentration of drug in μgm. per ml.	Neomycin	Q-19	Aureomycin	Chloromycetin
0.5				
1.0				
1.9	6			14,16
3.9	3,12,18			6,13,20
7.8	1,4,5,7,8,11, 13,14,16		6,14	3,7,8,11, 15,18
15.6	2,9,10,15		1,18	2,4,5,17
31.2	17,20		16	9,10,19
>31.2		1,2,3,4,5,6,7, 8,9,10,11,12, 13,14,15,16, 17,18,19,20	2,3,4,5,7,9,10, 11,12,13,15, 16,17,19,20	

Aerobacter

Concentration of drug in μgm. per ml.	Neomycin	Q-19	Aureomycin	Chloromycetin	Polymyxin B
0.5		3,5,6,14,			16
1.0	3,4,15,16,	15,16,20		1	14
1.9	17,19,20	1,4,11 7,10,13	4,16,17	5	5,11,13,15
3.9	1,2,6,11,14	2,9,17,19	2,5,6	6,7,13,16	7,10
7.8	13		3,7,13,14, 15,19,20	2,4,11,19	6,9
15.6	5,7,9,12			3,8,9,10,12, 14,15,18,20	
31.2	8,10			17	
>31.2	18	8,12,18	1,8,9,10, 11,12,18		8,12

FIG. 1.

Comparative action of neomycin, Q-19, chloromycetin, aureomycin, and polymyxin B against 20 strains each of *E. coli*, *P. aeruginosa*, *P. vulgaris*, and *A. aerogenes*. Strains are numbered from 1 to 20, and the sensitivities of each strain are denoted by the numerals in the columns over the names of the antibiotics.

TABLE I.
Number of Variants of *E. coli* Per 1 × 10^{10} Organisms Resistant to Various Concentrations of Antibiotics.

	Conc. of drug in μg per ml								
Antibiotic	3	5	6	10	12	25	50	100	1000
				Streptomycin					
Exp.									
1 a*			31		55	51	44	31	16
b			9		51	55	40	45	23
2 a		60		109		111	111	94	49
b		54		89			106	111	57
3 a	33	OG		60				83	
b	16	OG		81				81	
4 a	OG		23		139	73	81	42	18
b	OG		42		53	73	63	77	16
				Neomycin					
1 a			46		0	0	0	0	0
b			81		0	0	0	0	0
2 a		103		0		0	0	0	0
b		59		0		0	0	0	0
3 a	OG	20		0				0	
b	OG	24		0				0	
4 a	OG		62		0	0	0	0	0
b	OG		79		0	0	0	0	0
				Q-19					
2 a	OG	OG		OG		0		0	0
b		OG		OG		0		0	0
3 a	OG	63		0				0	
b	OG	87		0		0		0	
4 a	OG		OG		10	0	0	0	0
b	OG		OG				0	0	0

* Two plates were used with every conc. of drug in each exp. They are labeled a and b.
OG = Overgrown.

myxin B were the most effective antibiotics against this species (Fig. 1). Neomycin was less effective and aureomycin and chloromycetin were relatively inactive.

Proteus vulgaris—Neomycin and chloromycetin appeared most active against *P. vulgaris* (Fig. 1). Strain 19, which was resistant to neomycin, was sensitive to chloromycetin and strain 1, which was resistant to chloromycetin, was sensitive to neomycin. None of the 20 strains was affected by Q-19 and only 5 were moderately sensitive to aureomycin.

Aerobacter aerogenes—This species was similar to *E. coli* in that most of the strains were sensitive to the 5 antibiotics. Q-19 was the most effective drug against 14 strains. Seven strains were resistant to aureomycin, 3 to Q-19, 2 to polymyxin B, and 1 to neomycin. Two of the strains (8 and 12) unaffected by Q-19 also were not inhibited by polymyxin B.

Pattern of Resistant Variants. Studies with the stock strain of *E. coli* showed the same pattern of resistance to streptomycin that has been reported(11-14). In populations of *E. coli* greater than 1 x 10^{10}, resistant variants appeared in concentrations of streptomycin as high as 1,000 μg/ml (Table I). The same strain of *E. coli* displayed the "penicillin pattern" of resistance to both Q-19 and neomycin in which resistant variants appear at slightly higher concentrations of drugs than that which inhibits the majority of organisms, but none are found initially resistant to high concentrations of drug. Thus, a considerable number of resistant variants grew out in concentrations as high as 1000 μg/ml of streptomycin while none were found in concentrations higher than

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12. Meads, M., *J. Immunol.*, 1949, v63, 1.
13. Alexander, H., *Pediatrics*, 1949, v3, 227.
14. Newcombe, H. B., and Hawirko, R., *J. Bact.*, 1949, v57, 565.

TABLE II.
Bactericidal Activity of Neomycin and Q-19 Against *E. coli*.

Time after inoculation	Control No. of bacteria	20 μg/ml neomycin No. of bacteria	20 μg/ml Q-19 No. of bacteria
1 min.	2.3×10^6	4.2×10^6	2.0×10^6
40 ”	2.1×10^6	5.0×10^5	1.1×10^4
2 hr	3.9×10^6	5.0×10^1	2.4×10^3
4 ”	1.7×10^8	No growth	No growth
18 ”	3.3×10^8	” ”	” ”

12 μg/ml of Q-19 or neomycin.

Bactericidal or Bacteriostatic Activity of the Antibiotics: The bactericidal and bacteriostatic results with Q-19 and neomycin were essentially the same in 9 of 11 strains. When 2 x 10^6 *E. coli* were inoculated into broth containing 20 μg/ml of Q-19 and neomycin, the number of organisms dropped significantly in 40 minutes and the end of 4 hours, no viable bacteria were recovered (Table II). In the concentration of drugs employed, the action of Q-19 and neomycin was primarily bactericidal, whereas the action of aureomycin and chloromycetin is bacteriostatic. Thus, these drugs are similar to streptomycin in their bactericidal action but resemble penicillin and aureomycin in regard to the pattern of resistant variants which appear when a culture of *E. coli* is exposed to them.

Discussion. While *in vitro* results do not always coincide with clinical experience, the foregoing observations with neomycin, Q-19, and polymyxin B suggests that they may be useful in the therapy of some infections caused by *P. aeruginosa*, *P. vulgaris*, *A. aerogenes*, and *E. coli*. The great *in vitro* activity of Q-19 and polymyxin B against *P. aeruginosa* suggests that these drugs may be effective against infections caused by this organism. Similarly, neomycin and chloromycetin appear worthy of trial against infections due to *P. vulgaris*. It is encouraging that 79 of 80 strains of gram negative bacilli tested were sensitive to at least 1 of the 5 antibiotics, and the majority of strains relatively resistant to some of the antibiotics were highly sensitive to others. This might warrant the simultaneous use of 2 or more antibiotics when it is impractical to determine specific sensitivities of bacteria. Seventy-five of the 80 strains included in the 4 species were inhibited by 31.2 μg per ml of neomycin, which is a concentration of drug readily obtained in the urine following parenteral administration(15). The failure to demonstrate variants of *E. coli* highly resistant to either Q-19 or neomycin is evidence that the rapid appearance of resistant variants, which is a disadvantage with streptomycin therapy, might not occur during the clinical use of Q-19 or neomycin. These observations confirm the findings of Waksman in regard to the activity of crude neomycin(1).

The instability of aureomycin in dilute solutions makes it difficult to compare *in vitro* results over a period of 18 hours with those of neomycin, Q-19, and chloromycetin, which form stable solutions. The data show, however, that aureomycin is not as active against *P. vulgaris* and *P. aeruginosa* as it is against *E. coli* and *A. aerogenes*, and that it is not equally active against different strains of the 2 latter species.

The suggested chemical relationship between Q-19 and the group of antibiotics, which include polymyxin and aerosporin, seems to be borne out in the data presented (3). Two strains of *A. aerogenes* resistant to Q-19 were also resistant to polymyxin B and, in general, there was close agreement between the sensitivities of the strains to these 2 antibiotics. Clinical studies based on these findings are now in progress.

Summary and conclusions. Twenty strains each of *E. coli*, *Aerobacter aerogenes*, *Pseudomonas*, and *Proteus vulgaris* were tested for sensitivity to neomycin, Q-19, aureomycin, chloromycetin, and polymyxin B. Q-19 and polymyxin B were particularly effective against Pseudomonas. Neomycin and chloromycetin were most effective against Proteus.

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15. Unpublished data.

Only 5 of the 80 strains tested were not sensitive to 31.2 μg or less of neomycin.

The degree of resistance possessed by variants exposed to Q-19 and neomycin is low. These variants display the "penicillin" rather than the "streptomycin" type of drug resistance.

Q-19 and neomycin are bactericidal in a concentration equal to or only slightly higher than that in which they are bacteriostatic.

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Content of Desoxyribo and Ribonucleic Acids of Retina Under Various Conditions.* (17802)

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Reports from various sources indicate(1) that the cells of cortical centers show considerable loss of ribonucleic acid after intense physiological stimulation, the level of ribonucleic acid returning to normal only slowly. In these experiments the ribonucleic acid was determined by microscopic measurement of the ultraviolet absorption of individual cells. The interpretation of results of such measurements has recently been criticized(2). The retina consists of several layers of ganglion cells and their neurites and nerve cells differentiated to cones and rods. All these nerve cells are readily accessible, can be obtained in considerable amount and can easily be stimulated by physiological means and in varied intensity. We have attempted to study, by chemical methods, the influence of light stimuli on the ribonucleic acid content of the retina of the rabbit, but observed no significant effect. However, it was found that the retina differs with respect to the composition of its nucleic acid fraction considerably from other tissues (including the cortex of the brain.) As this research cannot be continued in the near future we report here our experimental material so far obtained.

Experimental preparation of materials. The eyes were enucleated immediately after death of the animal. To obtain easy and complete separation of the retina from the choroidea the eye was immediately placed in solid CO_2 and either was kept frozen for several days or, after being frozen, immersed in absolute alcohol at 0° and kept there for 5 days. Neither the freezing nor the treatment with alcohol interfered with the determination of the nucleic acids. The retina could be separated from the choroidea equally readily after being thawed and after fixation in alcohol.

Analytical methods. The nucleic acids were extracted according to the procedure of Schneider(3). Desoxyribonucleic acid (DNA) was determined as a rule by the diphenylamine reaction(4). Readings were carried out at 610 mμ with the Beckman spectrophotometer. To ascertain that the substance determined by the diphenylamine reaction was identical with desoxyribonucleic acid, the determination of the latter was carried out in a few cases by the cysteine reaction according to the procedure recommended by Stumpf (5). These completely different procedures gave practically identical results. The ribonucleic acid (RNA) was in all cases determined by the orcinol reaction according to

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* This work was supported by research grant to Dr. H. Waelsch from the Supreme Council 33rd Northern Masonic Jurisdiction, United States.

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5. Stumpf, P. K., *J. Biol. Chem.*, 1947, v169, 367.

TABLE I.
Ratio DNA/RNA in Retinas and Other Tissues of Various Species.

Exp. No.	Animal	Tissue	DNA[*] mg%	RNA[†] mg%	Ratio DNA/RNA
1	Rabbit	Retina	520	145	3.6
2	”	”	1240	232	5.3
3	Steer	”	530	130	4.1
4	”	”	692	200	3.5
5	”	”	392	140	2.8
6	”	”	460	140	3.2
7	”	”	396	95.4	4.1
8	Mouse	Liver	142	960	.15
9	”	”	178.5	810	.22
10	Cat	Grey Matter	120	130	.92
11	”	” ”	100	127	.78
12	Rabbit	” ”	71	124	.57
13	Steer	Optic Nerve	44	90	.49
14	”	” ”	41	76	.54
15	Rabbit	” ”	41	84.8	.48
16	Cat	Retina	824	206	4.0
17	”	”	1724	250	6.9
18	”	”	1750	250	7.0
19	”	”	1640	292	5.6
20	”	”	1568	292	5.3

[*] DNA—Desoxyribonucleic acid.
[†] RNA—Ribonucleic acid.

Dische and Schwartz(6). The results of these determinations were again checked in a few cases by the so-called basic cysteine reaction(7). Good agreement was found between both procedures. Nitrogen determinations were carried out by nesslerisation on an aliquot of the trichloracetic acid extract containing nucleic acids and on total residue of the TCA extraction. In the first series of experiments the DNA and RNA content of the retinas of steer, cat and rabbit was determined. The results of these experiments are listed in Table I. The values of nucleic acids were calculated either on the basis of wet weight of the retina (without pigment epithelium) (Exp. 1-15, Table I) or on the basis of the weight of the retina fixed by alcohol. (Exp. 16-20 Table I). Under these circumstances only the ratio DNA/RNA can be regarded as significant and comparable with the results of other tissues.

As can be seen from Table I DNA/RNA is smaller than 1 in the liver,[†] the gray matter and the optical nerve, while it varies between 2.1 and 7 in the retina of all species investigated.

In a second series of experiments (Table II) the content of DNA and RNA in retinas of rabbits kept dark 12-24 hours were compared with that of retinas exposed to light under various conditions. Three types of experiments were carried out. In the first type one eye of the animal was covered with a rubber cup tied by adhesive, while the other eye was exposed for 4 hours to daylight. (Exp. I) In a second experiment a group of 4 chinchilla rabbits was kept in dark for 8 hours while another group of 4 was exposed to daylight for the same period of time. (Exp. II) In a third experiment 2 groups of 3 chinchilla rabbits were kept over night in complete darkness and then one group was killed and the eyes immediately enucleated and frozen in dry ice while the other was exposed for 10 minutes to the light of a 150 Watt bulb at a distance of 10 cm before death. The globes were fixed in cold alcohol and DNA, RNA and nitrogen of the fixed retinas, (separated from pigment epithelium), determined. The DNA and RNA

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6. Dische, Z., and Schwartz, K., *Mikrochim. acta*, 1937, v2, 13.
7. Dische, Z., *J. Biol. Chem.*, 1949, v181, 379.

[†] The nucleic acid content of liver is well known and our determinations in liver were carried out, to check the reliability of our procedure for the determination of nucleic acid in grey matter and retina.

TABLE II.
DNA and RNA Content of Irradiated and Dark Adapted Retinas of Rabbits. All results expressed in mg of DNA, RNA per 100 mg Nitrogen.

Exp. No.	Stimulated by light DNA	Stimulated by light RNA	Dark adapted DNA	Dark adapted RNA
I	101	17.6	95	15.6
II	90	26	90	20
IIIa	130	29	130	38
IIIb	94.1	26	91.5	28

content was calculated per 100 mg nitrogen. As can be seen from Table II no significant differences were found between the dark adapted retinas and those exposed to light. The high ratio DNA/RNA in the retina as compared with other tissues is due partly to a very high content in DNA and partly relatively low values in RNA.

Discussion. It seems doubtful whether it is possible to explain the high ratio DNA/RNA by a higher ratio nucleus: cytoplasm in the retina as compared with the grey substance. The high ratio could be brought about by a much higher concentration of DNA in the nuclei of the retina or be due to a replacement of one part of RNA by DNA in the cytoplasm of the retina cells. The consideration of certain characteristic properties of nucleic acids shows that this latter alternative could be of significance for the understanding of certain correlations between structural factors and function of the retina. Nucleic acids can combine not only with histones in nucleoproteins, but can form labile complexes with neutral proteins in the neighborhood of their isoelectric point. Because of strong polarity, high degree of polymerisation and asymmetry of the molecules of nucleic acids they can, by combining with proteins, influence the insolubility as well as impart to the protein molecules specific spatial orientation. This spatial orientation of protein molecules can be regarded as an important factor in the structural integration of cellular constituents. This function of nucleic acids can be deduced not only from the role of desoxyribonucleic acids as constituent of genes but also from the correlation found by various authors between the amount of ribonucleic acid in embryonal and tumor cells and the speed of growth and differentiation. The desoxyribonucleic acid in cells seems to be more highly polymerized than ribonucleic acid. The orientating influence on protein exerted by nucleic acids in general will depend on their degree of polymerisation. The higher ratio DNA/RNA in the retina as compared with other tissues may indicate that certain structural factors in the cytoplasm underlying the specific function of the retina require nucleic acids of higher degree of polymerisation, as that of ribonucleic acid.

Summary. (1) The ratio of desoxyribonucleic acid:ribonucleic acid in mammalian retinas is reversed in comparison with that in grey matter and other tissues. (2) No differences were found in the content in ribonucleic acid between dark and light adapted retinas.

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Effect of Adrenalectomy on Spontaneous and Induced Proteinuria in the Rat.* (17803)

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Addis, Barrett, Boyd, and Ureen(1) have demonstrated that an intense transient proteinuria follows the intraperitoneal injection of the kidney enzyme renin into the normal rat. Addis and Boyd(2) state that the proteinuria following renin injection is abolished in the rat by bilateral adrenalectomy. In the course of our studies on the mechanism of proteinuria, we have confirmed and extended these observations and have demonstrated that treatment not only with aqueous adrenal cortex extract (ACE), but also with synthetic desoxycorticosterone acetate (DOCA), and 11-dehydro-17-hydroxy-corticosterone acetate (cortisone) will restore the ability of the adrenalectomized rat to respond to renin with a marked proteinuria. The effect of adrenalectomy on proteinuria produced by the intraperitoneal injection of human serum albumin, as well as on the spontaneous, slight proteinuria present in the normal rat have also been investigated.

Methods. Two hundred seventeen male and female albino rats of the Slonaker strain were used in these experiments. Proteinuria in response to renin injection was determined on the fifth and tenth postoperative days in the adrenalectomized and control animals. Four dog units(3) of hog renin diluted in 4 cc of physiological saline solution were given intraperitoneally. The animals were then placed in individual metabolism cages, and the urine excreted over the ensuing 60 minutes was carefully collected. The methods for accurate rat urine collections are described in detail by Addis, *et al.*(1). Animals given hormone replacement therapy received daily injections from the day of operation until the termination of the experiment. The dosage schedule was as follows: desoxycorticosterone acetate, 0.5 mg subcutaneously; adrenal cortex extract, 2 cc intraperitoneally; and cortisone, 1.0 mg subcutaneously daily. Serum albumin proteinuria was produced by injecting rats intraperitoneally with 16 cc of 6% human serum albumin§ in normal saline solution, according to 2 dosage schedules. Group A, consisting of 6 adrenalectomized and 4 control animals, received 16 cc of 6% serum albumin in normal saline solution intraperitoneally at 9:00 a.m. and 4:00 p.m. on the fourth postoperative day, and at 10:00 a.m. on the fifth postoperative day. (A total of 3 g of albumin.) They were then placed in individual metabolism cages, and urine was collected over the ensuing 18-hour period.

Group B, consisting of 8 adrenalectomized and 4 control animals, received this dose at 9:00 a.m. and 4:00 p.m. on the fifth postoperative day (a total of 2 g of albumin). They were then placed in individual metabolism cages, and their urine was collected during the following 18-hour, overnight period. Adrenalectomies were performed through bilateral flank incisions uring ether anesthesia. All adrenalectomized rats were maintained on normal saline drinking solution. Urinary

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[*] This work was made possible by grants from the United States Public Health Service and The American Foundation for High Blood Pressure.

Desoxycorticosterone acetate was supplied by Dr. G. Babcock, Jr. of Schering Corp., Bloomfield, N.J.

Adrenal cortex extract was supplied by Dr. David Klein of The Wilson Laboratories, Chicago, Ill.

[†] Dr. Addis' untimely death occurred before this paper was completed.

[‡] Columbia Foundation Research Fellow.

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[§] Obtained through the courtesy of the American Red Cross.

TABLE I.
Abolition of Renin Proteinuria by Adrenalectomy and Its Restoration by Adrenal Hormone Therapy.

	No. of observations	Renin proteinuria, mg/hr
Normal controls	96	31.7
Sham operated controls	75	37.7
Adrenalectomy	50	1.0
,, + DOCA	12	33.1
,, + ACE	14	16.5
,, + Compound E	23	33.5

proteins were determined by a modification of Kingsley's biuret method(1).

Results. (A) *Effect of Adrenalectomy on Renin Induced Proteinuria.*

All rats excrete protein in their urine. In the normal male rat this proteinuria may amount to 1.0 mg per hour. In the hour following the intraperitoneal injection of 4 dog units of renin, the protein excretion increases markedly. While there is a great deal of individual variation in the amount of protein excreted during this hour, the average in 96 animals shown in Table I is 31.7 mg. In view of the variability in protein excretion, the value of 37.7 mg/hr for the sham operated control is not significantly greater than the normal control value. The data shown in Table I indicate that bilateral adrenalectomy renders the saline-maintained rat incapable of responding to renin injection with significant proteinuria.

(B) *Renin Proteinuria in Adrenalectomized Rat Receiving Adrenal Cortex Steroids.* Both desoxycorticosterone acetate and cortisone restore the ability of the adrenalectomized rat to respond to renin injection with massive proteinuria (Table I). Adrenal cortex extract does not seem to restore this function as completely as the other compounds in the dosage used in these experiments.

(C) *Human Serum Albumin Proteinuria.* The normal rat excretes about 510 mg of protein in the 18 hours following the last intraperitoneal injection of human serum albumin. After bilateral adrenalectomy, 50-100 mg of protein are excreted in the 18

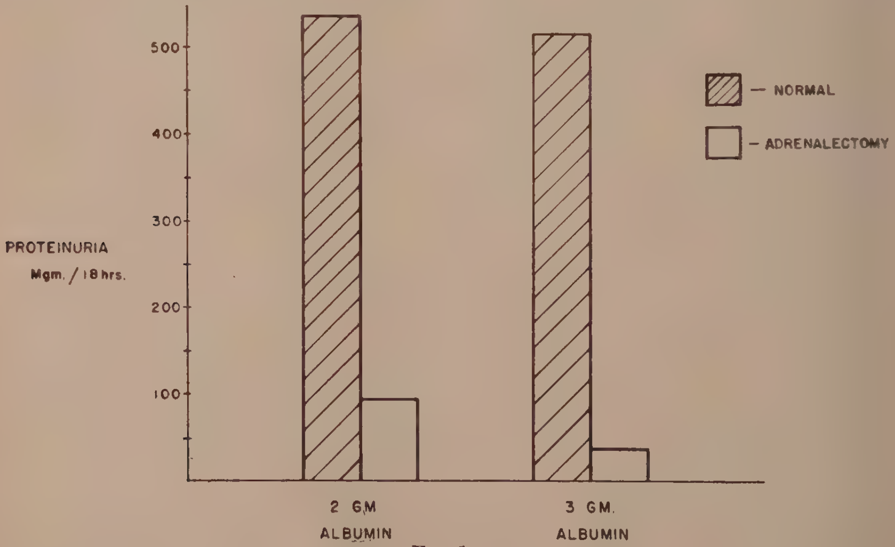

FIG. 1.
Effect of adrenalectomy on the proteinuria produced by the intraperitoneal injection of human serum albumin.

TABLE II.
Effect of Adrenalectomy and Adrenal Steroid Therapy on Spontaneous Proteinuria of Male and Female Rats.

	Male			Female	
	No. of observations	Mean proteinuria mg/hr/100 cm^2 body surface	Stand. dev.	No. of observations	Mean proteinuria mg/hr/100 cm^2 body surface
Normal controls	51	.19*	.052	75	.10
Adrenalectomy	25	.11*	.052	44	.08
'' + Cortisone	23	.19*	.071	18	.10
'' + DOCA	12	.16*	.035	18	.07
'' + ACE	12	.12	.035	24	.06

* The difference between the means of the normal controls, adrenalectomy plus cortisone, and adrenalectomy plus DOCA and the mean of the untreated adrenalectomized animals exceeds three times the standard error of the difference between these means.

hours following the albumin injections. As with renin, there is great variability in the amount of protein individual animals excrete following albumin injection. This variability is not indicated by the averages shown in Fig. 1. Nevertheless, the effect of adrenalectomy in decreasing this proteinuria is so clear cut and striking as to be beyond doubt.

Normal male rats have a higher rate of spontaneous protein excretion in their urine than normal female rats(4). (Table II). The data in Table II indicate that bilateral adrenalectomy decreases the rate of spontaneous proteinuria in the normal male rat. The results have been expressed in mg/hr/100 sq. cm. of body suface in order to allow comparison of animals with different body weights and kidney mass. A statistical analysis of these results shows that the decrease in proteinuria in the male rat following adrenalectomy is a significant one.

The administration of cortisone in dosage of 1 mg subcutaneously daily restored the spontaneous protein excretion of adrenalectomized male rats to normal values within 5 days. Desoxycorticosterone acetate (0.5 mg daily) proved less adequate in this regard, while adrenal cortex extract (2 cc intraperitoneally daily) did not increase the protein excretion over that found in the untreated controls. Bilateral adrenalectomy apparently does not alter the level of normal spontaneous proteinuria in the female rat. (Table II).

Discussion. Our data establish a relationship between the adrenal cortex and protein excretion by the kidney, but do not indicate the mechanism of this interaction. It is possible that adrenalectomy decreases protein excretion by altering the renal blood flow, or decreasing the rate of glomerular filtration of protein. Lotspeich(6) and Friedman, Mackenzie, and Friedman(7) have reported essentially normal rates of glomerular filtration in the adrenalectomized rat maintained on saline solution. The later workers have demonstrated that renal blood flow is unaltered in the saline treated, adrenalectomized rat when studied 4-6 days postoperatively. While the urinary output of adrenalectomized rats is less than that of normal controls, Lotspeich(6) maintains that this is due to an increased tubular reabsorption of water. Small changes in these renal functions could be responsible for the fall in spontaneous protein excretion found in the adrenalectomized male rats, but could hardly account for the gross decreases in renin and human albumin proteinuria following bilateral adrenalectomy. The effect of adrenalectomy

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5. Addis, T., Final Report, Contract 338, Office Of Scientific Research and Development, Committee on Medical Research, 1946.

6. Lotspeich, W. D., *Endocrinology*, 1949, v44, 314.

7. Friedman, S. M., Mackenzie, K. R., and Friedman, C. L., *Endocrinology*, 1948, v43, 123.

on the glomerular permeability to protein, as well as on the tubular reabsorption of protein is now under investigation.

Summary. 1. Bilateral adrenalectomy abolishes or greatly reduces the types of experimentally produced proteinuria studied in the saline maintained rat.

2. The normal, spontaneous proteinuria of the male rat is significantly reduced by bilateral adrenalectomy.

3. Cortisone administration to bilaterally adrenalectomized male rats increases their spontaneous proteinuria to normal control levels.

4. Adrenal cortex extract, desoxycorticosterone acetate, and cortisone restore the ability of the salt-maintained, adrenalectomized rat to respond to renin injection with massive proteinuria.

We are indebted to Drs. Harry Goldblatt, Erwin Haas, and Hildegarde Lamfrom for supplying us with the renin used in this study.

We wish to thank Mr. J. Manders and Mr. A. Mallinger for their technical aid.

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The Dermal Loss of Iron.* (17804)

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(Introduced by William G. Clark.)

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It has been generally accepted that the human being has a limited capacity to lose iron except by hemorrhage. Voluminous data obtained from careful iron balance studies have indicated that: (a) very little dietary iron will keep a normal individual in iron balance; (b) the loss of iron in the urine and stool amounts to no more than 1 mg per day; (c) only a small amount of iron is absorbed from the gastro-intestinal tract even when iron is given in large amounts; and (d) the body supply of iron, once across the intestinal barrier, is closely conserved(1-5). The findings of Mitchell and Hamilton(6) who report as much as 6.5 mg of iron lost in sweat per day indicate a hitherto unsuspected avenue of iron loss.

This paper presents the results of 118 determinations of iron in sweat obtained from 25 normal subjects.

Method. 1. The method of Leslie and Levin(7), used in obtaining the sweat sample, consists essentially of carefully washing one forearm with soap and water, rinsing with copious amounts of distilled water, and drying carefully with specially cleaned towels. Finger cots are applied to preclude fingernail contamination and a full-length rubber glove is then drawn over the hand and forearm to catch the sweat. The other arm is placed in a water bath at 110° F for one hour. In most instances a sufficient volume of sweat is obtained. The sweat which accumulates in the glove is measured in a graduated centri-

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* Sponsored by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions of the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

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TABLE I.
Determinations of Iron in Sweat.

No. of samples per subject	Avg volume of sweat collected, cc	In "cell poor" sweat, µg per cc	In "cell rich" sweat, µg per cc
4*	9.3	.31	8.45
1	15.	.18	19.5
3	16.	.51	2.56
3	14.7	.26	3.63
3	9.7	.64	10.73
8	12.3	.30	6.94
2	10.5	2.15	17.2
5	10.4	.52	3.74
1	5.	.00	4.9
2	10.	.00	4.2
1	5.	.00	6.1
1	5.	.00	4.5
1	6.	.00	2.2
1	14.	.00	9.5
8	10.1	.23	5.3
1	15.	.00	4.1
1	4.	.00	8.3
1	5.	.00	10.4
2	11.8	.38	9.
2	19.5	.45	.78
1	20.	.00	4.9
1	7.7	.55	9.5
1	5.	.20	4.3
4	8.1	.19	4.75
1	7.5	.55	10.9

* Where more than one sweat sample per subject was analyzed, figures represent averages.

fuge tube and the volume recorded.

Iron analyses of distilled water rinsings and arm washings were made in order to be sure that iron was not introduced erroneously into the sweat sample. All subjects were in a fasting state before the sweat was obtained. Two glasses of water were drunk before the sweating period.

2. Iron determinations were made using an ortho-phenanthrolin method(8-12), modified for these purposes. In this method iron is reduced by hydroquinone to the ferrous state and unites with ortho-phenanthrolin in a solution of pH 3.5-4.5 to produce a pink color. The solution is buffered with ammonium acetate. The procedure is carried out as follows: To a 2 cc aliquot of the sample in a 20 cc test tube are added one cc of C. P. concentrated sulfuric acid and 2 glass beads. The test tube is then heated over a micro burner, care being taken to avoid loss from bumping. The digestion usually takes about 15 minutes. The solution is then cooled and enough 30% hydrogen peroxide is added dropwise until clear. The sample is then re-heated to boiling to destroy the excess H_2O_2, after which the total volume is re-adjusted to 2.0 cc with distilled water. To the tube are added, in order, the following solutions: 0.3 cc of 1% hydroquinone (freshly prepared), 1.0 cc of 0.1% ortho-phenanthrolin and 7.0 cc of 50% ammonium acetate. The tubes are shaken vigorously. Color is allowed to develop for one hour. It is then read in a colorimeter (the Klett-Summerson photoelectric colorimeter with light filter No. 50 was used in these experiments). Distilled water blanks and standards containing .002 mg of iron per cc were run simultaneously. Using this method, we were able to recover 98% of known amounts of added iron. However, great caution must be exercised to avoid

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the introduction of iron, either by accident or by contaminated chemicals.

Discussion of results. The analyses of "cell rich" and "cell poor" sweat from 25 different normal subjects are given in Table I.

"Cell rich" indicates sweat just as it came from the glove. "Cell poor" refers to the supernatant obtained after centrifugation. The former material was opalescent, the latter clear. By far the larger amount of iron was present in the "cell rich" sweat. With one exception all determinations of "cell poor" sweat showed less than 0.64 μg of iron per cc, which is below the limits of accuracy of this method.

In order to check the results further a rubber glove was applied to a subject's arm after the usual preparation. Distilled water was allowed to remain in contact with the arm for one hour and the contents of the glove were then analyzed for iron. The material obtained was opalescent and rich in epithelial cells; it yielded an iron content of 8.5 μg/cc, corresponding to "cell rich" sweat. The supernatant after centrifugation gave a blank reading for iron. This has been repeated several times with similar results.

From the data obtained it is concluded that the loss of iron in human sweat is negligible. Loss of iron from desquamation of the epithelium may be considerable, although no quantitative estimates were made. Further iron balance work with reference to the loss of iron by means of desquamation of epithelial cells is indicated.

Summary. "Cell poor" and "cell rich" sweat samples from 25 normal subjects have been analyzed (total of 118 determinations). The iron content of "cell poor" sweat was negligible. On the other hand "cell rich" sweat contained a high concentration of iron. This iron loss is not an essential function of the process of sweating, but is associated primarily with desquamation.

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Effect of DDT on Testes and Secondary Sex Characters of White Leghorn Cockerels. (17805)

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With the introduction of the insecticide DDT (2,2-Bis(p-chlorophenyl) 1,1,1-Trichloroethane) and its subsequent widespread application, there came a need for a complete understanding of its toxicity, and metabolic effects in animals other than insects. Studies on several species of mammals have resulted in a rather wide variety of findings regarding the action of this compound. In general, mammals are extremely sensitive to relatively small amounts of DDT. On the other hand, birds have been shown to possess a high degree of resistance to this substance(1). Since experiments dealing with the effects of DDT on birds are notably less numerous than similar studies on mammals, it appeared that additional information regarding this important compound might be obtained from a study designed to reveal the effects of long-term poisoning in chickens. In the course of this study on cockerels, some interesting effects were noted on the development of the testes and secondary sex characters.

Method. White Leghorn cockerel chicks were selected at random and divided into 2 groups, one to serve as controls and the other as experimental animals. They were placed in a commercial brooder and allowed free access to food and water. Two series of experiments were carried out involving a total of 30 controls and 40 treated birds. Starting on the 8th day after hatching, the experimental chicks were given daily subcutaneous injections of purified DDT (M.P. 109°). To

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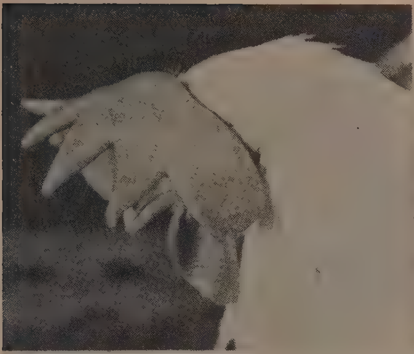

FIG. 1

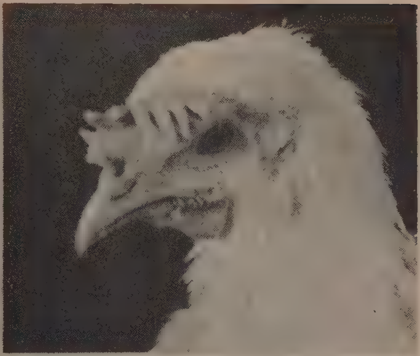

FIG. 2

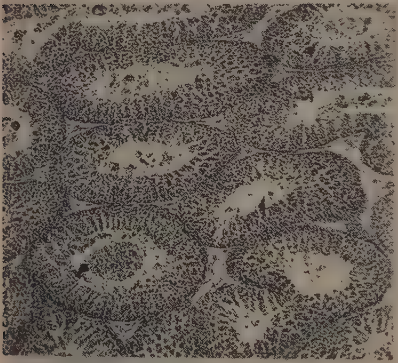

FIG. 4

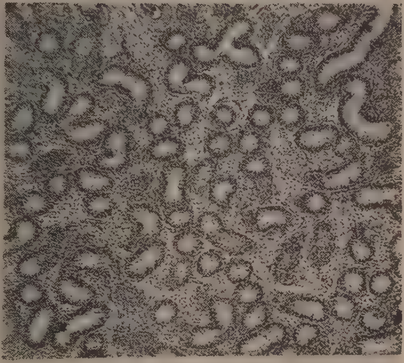

FIG. 5

FIG. 1. Control bird, age 60 days.
FIG. 2. Treated bird, same age.
FIG. 4. Section of testis from control bird (× 60), age 86 days.
FIG. 5. Section of testis from treated bird (× 60), same age.

minimize the possibility of lesions, the site of injection was varied. The amount of the compound administered was gradually increased during the course of the experiment from an initial dose of 15 mg per kilo of body weight in the young birds to a maximum of 300 mg per kilo near the terminal stages of the study. The solvent used was rendered chicken fat. Control birds received an equivalent amount of the fat each time the treated birds were injected. All birds were treated for at least 60 days and some were carried for as long as 89 days. Careful weight records were kept throughout the course of the study. At the termination of the experiment the animals were sacrificed and the testes removed, weighed and prepared for sectioning.

Results and discussion. As treatment proceeded, it became increasingly evident that there were significant differences in the development of the secondary sex characters between the two groups. After about 25 days of treatment, the experimental animals began to display marked inhibition of combs and wattles both in regard to size and color. As the experiment progressed, these differences became more striking. Fig. 1 and 2 serve to illustrate the effects after 52 days of treatment. At this stage comb measurements were made which further demonstrated the difference between the two groups. The aver-

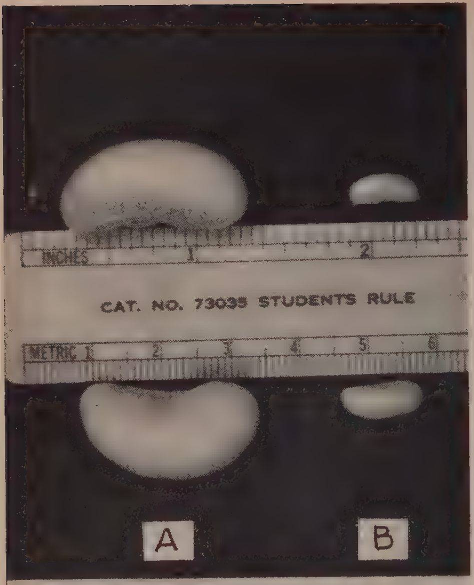

FIG. 3

Showing the comparative size of testes from normal bird (A) and those from DDT-treated animal (B) of the same age. (85 days).

age comb size (taken as length plus height) for the control group was 11.7 cm as compared to an average of 7.56 cm for the experimental birds. A few instances of comb measurements made after 80 days of treatment were even more striking, the control combs measuring 21.3 cm and treated, 8.9 cm. These observations led us to suspect an involvement of the testes in the production of this phenomenon, since it is well known that atrophy of head furnishings results from castration of Leghorn cockerels.

Therefore, when the birds were sacrificed, first attention was given to the stage of development of the testes. In a group which was killed at an age of between 80 and 90 days, the average testes weight of the treated animals was 1.05 g, while the average for the normals was 5.63 g. Fig. 3 serves to illustrate the relative size of the gonads from treated and untreated birds of the same age. Tissue sections were prepared of the testes of both treated and normal animals and these further emphasize the effect which was produced. Fig. 4 and 5 provide a means of comparison. Careful examination of the sections reveals that the amount of intertubular tissue in the organ from the treated bird is far in excess of that found in the control animal. In contrast to this observation it can be seen further that the tubular development has been markedly retarded in the treated testis.

In noting the results of the treatment, it was necessary to consider the possibility that the over-all effects did not occur as a result of a direct action of the DDT itself, but instead were the consequences of a generalized condition of poor health brought about by the presence of a toxic agent. It is not without significance that workers have attributed to the factor of inanition many of the results obtained by administration of the compound(2,3). In this study, no difference in food or water intake between the two groups was discernible from casual observation and the weight increments of the treated and control animals present a strikingly similar picture. The weights, when plotted for the entire course of the experiment, can be served by a single curve for both groups (Fig. 6). To further ascertain whether the poison was affecting the general health, red blood cell counts and hemoglobin measurements were made. The experimental birds were shown to have an average of 17.8% fewer red cells than the controls. This difference was not considered great enough to influence the general health. There were no significant differences in the hemoglobin values.

Attempts to explain the results obtained in this work are difficult since the literature does not contain any evidence which shows similar effects in other animals. There is one report however, which lists occasional testicular atrophy in rats as a result of DDT feeding experiments(1). No significance was placed on the finding since it occurred in only rare instances.

The effects noted here are such that they

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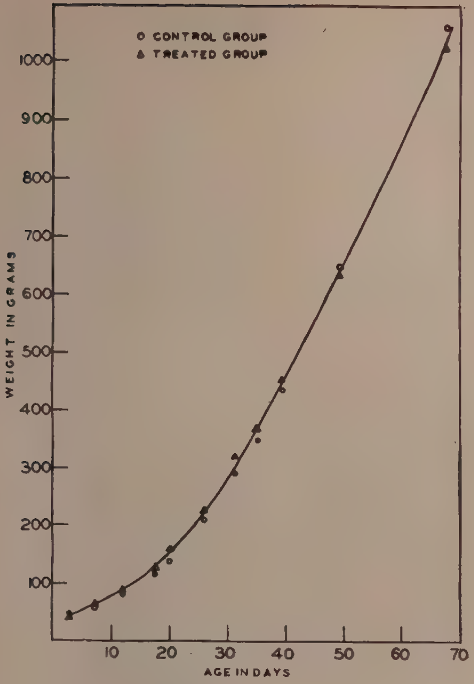
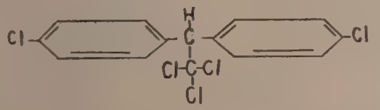
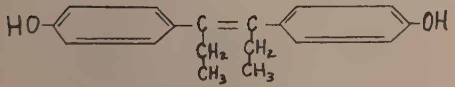


WEIGHT CURVE
FIG. 6

might easily be duplicated by the administration of an estrogen. It seems, therefore, that the possibility of an estrogenic action of DDT is at least worthy of consideration. In speculating along these lines, it is interesting to note the degree of similarity between the molecular configuration of DDT and certain synthetic estrogens, especially diethylstilbestrol.



DDT



Diethylstilbestrol

Similar chemical structures by no means insure a common biological action; however, it is interesting to note that introduction of chlorine into the stilbestrol molecule has been reported to result in a compound that still possesses some estrogenic activity(4). Assuming that DDT was acting in such a capacity, it is very possible that it might be doing so through an inhibition of pituitary gonadotrophins. Noble(5), among others, has reported a decreased gonadotropic content in rats treated with diethylstilbestrol. Experiments have been designed to determine whether or not DDT is acting in this fashion.

Another factor that must be considered is the manner in which chickens metabolize DDT. Perhaps there occurs a slight alteration in the structure of the compound which enables it to produce the effects noted. That chickens do handle the substance differently from mammals is suggested by the difference in tolerance levels exhibited. It is quite possible that doses of the same magnitude as those administered to chickens in this experiment might produce comparable effects upon mammalian testes. However, these levels are so high as to be fatal for most mammals.

Summary. 1. White Leghorn cockerels were subjected to injections of DDT beginning 8 days after hatching and continuing for as long as 89 days.

2. The development of the comb and wattles was greatly inhibited as a result of the treatment. This effect is attributed to the marked retardation of the testes growth which was noted.

3. These findings suggest that DDT may exert an estrogen-like action in white Leghorn cockerels.

We wish to express our appreciation to E. I. du Pont de Nemours and Co., for generously supplying the DDT used in this study.

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Received March 31, 1950. P.S.E.B.M., 1950, v74.

Synthesis of Citrovorum Factor From Folic Acid By Liver Slices; Augmentation By Ascorbic Acid.* (17806)

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An important role of ascorbic acid in the development of megaloblastic anemia has been indicated by various clinical observations, and these have been reviewed by Vilter (1) and by May, *et al.*(2). Studies of experimental deficiencies in the human infant(2), monkey(3), guinea pig(4), rat(5), and chick (6) have suggested that ascorbic acid is involved in the metabolism of pteroylglutamic acid (PGA). The studies described in this paper indicate that ascorbic acid may participate in the metabolic alteration of PGA to form a compound or compounds with remarkable activity for *Leuconostoc citrovorum* 8081. The citrovorum factor(s)(CF), first described by Sauberlich and Baumann(7), which is required for the growth of this organism, is considered to be closely related to PGA(8-12). In this report, further evidence for the relationship between the two factors is afforded by the observations that (i) the liver of PGA-deficient rats contains a greatly reduced amount of CF as well as of PGA, (ii) the enzymatic formation of CF by rat liver slices is greatly increased by the addition of PGA, and (iii) the yield of CF, under these conditions, is augmented significantly by the addition of ascorbic acid.

Experimental. Weanling albino rats (Sprague-Dawley-Holtzman strain) were depleted of PGA on a purified diet containing, in g per 100 g: "vitamin-free" casein (Labco) 18, cystine 0.2, glucose (Cerelose) 67.5, cellulose (Celluflour) 2, salts IV(13) 4, succinylsulfathiazole (Sulfasuxidine) 2, hydrogenated vegetable oil (Primex) 4, fortified corn oil (supplying 2200 I.U. vitamin A, 300 I.U. vitamin D, and 2.5 mg of α-tocopherol acetate) 2, choline chloride 0.1, and a mixture of B-vitamins 0.2 (supplying thiamine hydrochloride, riboflavin, and pyridoxine hydrochloride, each 0.5 mg, nicotinic acid and calcium pantothenate, each 2.0 mg, inositol 10 mg, and menadione 0.1 mg). After about 5 weeks on this diet, individual rats weighing from 80 to 100 g were selected for the tissue studies when the acute state of PGA-deficiency was indicated by rapid loss of body weight and severe diarrhea. The normal rats (av. wt. 200 g) were raised on a complete stock diet. The rats were stunned, decapitated, bled and the liver was removed. Liver slices, prepared with the Stadie-Riggs micro-

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* This investigation was supported, in part, by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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TABLE I.
Synthesis of Citrovorum Factor by Rat Liver Slices.

	Units of citrovorum factor per g of liver, fresh wt Additions to vessels containing liver slices*			
Tissue	None	Ascorbic acid, 10 mg	PGA, 100 μg	PGA, 100 μg, + ascorbic acid, 10 mg
Normal rat liver				
Exp. 4 (1)†	2880	—	5530	7500
,, 5 (1)	2220	4160	3850	5810
,, 5 (1)	1710	4375	3660	10930
PGA-deficient rat liver				
Exp. 1 (3)	147	215	3020	6340
,, 3 (5)	73	85	2500	4460
,, 4 (4)	36	—	4200	8700
PGA-deficient rat liver				
Exp. 2 (3)	88	190	3460	6600 (5 mg)‡ 8000 (10 mg)‡ 6500 (30 mg)‡

* Liver slices (1 g) were suspended in air-equilibrated Krebs-Ringer phosphate (5 ml). All vessels were incubated at 37° for 2 hrs in a constant temperature water bath with shaking.

† Each vessel contained slices from the number of animals indicated by the figure enclosed within parentheses.

‡ The figures expressed parenthetically indicate the amount of ascorbic acid added in these cases (PGA, 100 μg).

tome(14), were placed in consecutive beakers (20 ml) in regular sequence. The beakers, which contained chilled Krebs-Ringer phosphate (pH 7.4) and, where indicated, PGA, were weighed to the nearest 0.01 g before and after addition of the slices (approximately 1 g). In some cases freshly neutralized ascorbic acid was added immediately before the vessels were agitated for 2 hours in a covered, constant temperature (37°) water bath. The final volume of fluid in each beaker was 5 ml. After heating at 100° for 5 minutes the contents of each vessel were ground in a glass homogenizer. The mixture was transferred to a glass-stoppered test tube, adjusted to pH 6.5, diluted to 25 ml, steamed at 100° for 10 minutes, thoroughly mixed, and filtered. The clear filtrates were assayed microbiologically with *Leuconostoc citrovorum* 8081, using the basal medium described by Sauberlich and Baumann(7). Under the conditions of this assay, added PGA was so greatly diluted as to exert no determinable growth-promoting action on the microorganisms.† Turbidimetric readings were made after about 20 hours at 37° using the Klett-Summerson photoelectric colorimeter with a 660 mμ filter.

Results. The results, presented in Table I, are expressed in terms of units of CF activity.‡

The liver slices of normal rats were found to contain, after incubation for 2 hours, about 2000 units of CF activity per g, wet weight. This amount was doubled when incubation was carried out in the presence of PGA (100 μg) or in the presence of ascorbic acid

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† As has been shown elsewhere (Nichol, C. A., and Welch, A. D., *Fed. Proc.*, 1950, v9, 367) the response of *Leuconostoc citrovorum* to the factor(s) derived from PGA is potentiated by thymidine and other naturally occurring substances. However, so great were the dilutions of the liver tissue employed in the determinations of the high CF levels, that the findings recorded were not significantly influenced by these factors.

‡ Units of activity employed in this study cannot be compared directly with those used by other workers in this field where both titrimetric and turbidimetric assays have been used and reference standards have differed. In this laboratory a liver fraction is used as a constant reference standard; one unit is defined as that amount needed, per 10 ml assay tube, to produce half-maximal turbidity in 20 hours at 37°.

(10 mg), while a combination of PGA and ascorbic acid increased the level of CF 3- to 5-fold. Although considerable variation was noted in the amount of CF in individual rats, the differences between treatments showed a very consistent trend in the tissue replicates.

The use of PGA-deficient rats offered a real advantage in that only a small amount of CF was present or was produced during the 2-hour incubation of liver slices from these animals. Under these conditions ascorbic acid (10 mg) exerted little effect on the yield of CF, while the addition of PGA (100 μg) produced nearly as much CF as was found in the normal liver tissue after incubation with PGA; similarly, incubation with both PGA and ascorbic acid gave values for CF which were approximately double those obtained with PGA alone. The effect of ascorbic does not appear to be specific since it can be produced also by glucoascorbic acid. Extensive studies of other compounds with a high reducing potential have not yet been made, but glutathione under the conditions tested appears to be much less active. In contrast to slices, homogenates of normal or PGA-deficient rat liver form very little CF from added PGA.

Discussion. The results presented indicate clearly that enzyme systems are present in rat liver that are able to convert PGA to a substance highly active for *Leuconostoc citrovorum*. The favorable effect of ascorbic acid and glucoascorbic acid on this synthesis might suggest that CF is readily oxidized; however, concentrates of CF are remarkably stable, except in an acid medium, and do not appear to be attacked readily by atmospheric oxygen. It is possible that in the series of reactions involved in the conversion of PGA to CF, reduction may occur. Presumably, CF is more closely related in structure to a prosthetic group of which PGA serves as a precursor.

Although the microbiological potency of pure CF is not known, studies of concentrates (10) suggest that under the conditions of these experiments only a small fraction of the added PGA is converted to CF. Despite the fact that normal rat liver usually contains less than 10 μg of PGA per g, as determined with *Lactobacillus casei*(15), addition of ascorbic acid produced slightly more CF from materials present in this tissue than did the addition of 100 μg of PGA in the absence of ascorbate. Apparently the substances present in liver that augment the growth of *L. casei* are more readily converted to a substance or substances active for *Leuconostoc citrovorum* than is synthetic PGA.

The physiological significance of the augmentation by ascorbic acid of the synthesis of CF from PGA, is indicated also by studies now in progress in rats and in human subjects. Preliminary findings show that the urinary excretion of CF, which is augmented strikingly by the administration of PGA, is further increased from 2- to 4-fold by doses of ascorbic acid ranging from 10 to 20 times that of PGA.

The fact that ascorbic acid increases the formation of CF from PGA suggests the possibility that a severe deficiency of vitamin C might depress the metabolic alteration of PGA to CF. In keeping with this possibility is the demonstrated relationship of scurvy to the appearance of megaloblastic anemia in human infants and monkeys, a condition relieved by treatment with PGA (May, *et al.*)(2,3). Also, in another species unable to synthesize ascorbic acid, the guinea pig, a deficiency of vitamin C leads to an abnormality in the metabolism of tyrosine, a condition also relieved by relatively large doses of PGA(4). It is reasonable to postulate that the conversion of PGA to CF is limited by a severe deficiency of ascorbic acid, so that effective amounts of the essential derivative are no longer obtained from PGA unless the intake of folic acid is materially increased.

Summary. Rat liver slices, normal or folic acid-deficient, are shown to form in the presence of pteroylglutamic acid a factor required for the growth of *Leuconostoc citrovorum* 8081. Ascorbic acid augments significantly the yield of the factor. The rela-

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tionship of ascorbic acid to the metabolic activity of folic acid is discussed.

The authors are grateful to Marilyn F. Wilson and Caroline A. McKalen for their invaluable technical assistance.

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Procaine and Autonomic Innervation. (17807)

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(Introduced by C. Heymans.)
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Hunt(1) reported that procaine may counteract the influence of acetylcholine on blood pressure and heart rate. Hazard, Corteggiani and Cheymol(2) showed that intravenous injection of procaine paralyzes the cardiac vagus innervation and suppresses the bradycardia by acetylcholine(3), physostigmine(4) and nicotine(5), while the hypotensive effects of acetylcholine are maintained. The hypertensive effect of acetylcholine in atropinized animals may be changed to a hypotensive by procaine. Harvey(6) and Dutta(7) stated that procaine may block conduction in the first cervical sympathetic ganglion. Laqua (8) showed that procaine protects the guinea pig against physostigmine intoxication. Conway *et al.*(9) reported that physostigmine and di-isopropylfluorophosphate(DFP) protect mice against procaine intoxication, while neostigmine and carbamic acid, N,N-dimethyl- 4 -dimethylamino-3-isopropylphenyl ester, methiodide (DIP) lower the LD50 of procaine.

Experiments have been performed on 24 dogs anaesthetized with chloralosane to investigate the mechanism of action of procaine on autonomic innervation.

The observations may be summarized as follows: 1. Small doses of procaine (10-40 mg/kg) given intravenously to 11 atropinized dogs decrease acetylcholine (60-240 γ/kg) hyperpnea and change the hypertensive effect to an arterial hypotension, while the vasopressor reflexes of carotid sinus origin are unaltered.

2. High doses of procaine in 15 normal dogs (40-300 mg/kg) curarize first intercostal respiratory muscles, then the diaphragm and finally the skeletal muscles, and suppress bradycardia induced by vagus stimulation or by nicotine. Small doses of acetylcholine (30-60 γ/kg) provoke arterial hypotension, without bradycardia. Higher doses of acetylcholine still induce bradycardia, which is in turn suppressed by higher doses of procaine. Vasopressor reflexes of carotid sinus origin or induced by central vagus nerve stimulation are first depressed and then blocked by high doses of procaine. Vasomotor synaptic stimu-

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lation by nicotine is decreased by low doses and suppressed by higher doses of procaine.

3. Procaine (40-100 mg/kg) protects against physostigmine (0, 1-0, 3 mg/kg) and di-isopropylfluorophosphate (DFP) (10-40 mg/kg) bradycardia, bronchospasm, salivation, muscular fasciculations and convulsions.

4. After injection of doses of physostigmine, dimethyl-carbamate of hydroxyphenyl-benzyl-trimethylammonium (Nu 683) or DFP inducing bradycardia, bronchospasm, muscular fasciculations and convulsions, injection of procaine (40-100 mg/kg) suppresses these symptoms, although marked cholinergic reactions may still be induced by injection of acetylcholine. Procaine thus acts against physostigmine and DFP intoxication in the same way as tetra-ethyl-ammonium, diethylaminoethyl-ester of phenyl-cyclopentane-carboxylic acid (Parpanit) and diethylaminoethyl-pheno-thiazine (Diparcol)(10-13).

5. After injection of doses of procaine blocking the heart vagus innervation, suppressing the nicotinic and muscarinic action of acetylcholine on the heart and suppressing the nicotinic action of acetylcholine on sympathetic vasomotor synapses, injection of physostigmine (0.3-0.5 mg/kg) or DFP (5-10 mg/kg) deblocks the heart vagus innervation and restores the nicotinic and muscarinic effects of acetylcholine on the heart and on the vasomotor synapses. This antagonistic effect of physostigmine and DFP occurs when serum cholinesterase (pseudo-cholinesterase) is completely inactivated only, while acetylcholine-esterase (true-cholinesterase) of erythrocytes is only inhibited to 15-20% of normal values. The titropotentiometric method of Delaunois and Casier(14) has been used for determinations of cholinesterases activity.

After deblocking the effects of procaine by physostigmine or DFP, one may induce again the paralyzing effects of procaine by injection of higher doses of the drug, and in turn deblock these procaine blocking effects by new injections of physostigmine or DFP.

Conclusions. Procaine paralyzes first the parasympathetic and later the sympathetic synapses. Procaine suppresses the nicotinic and muscarinic effects of acetylcholine on the heart, but not the muscarinic effect of acetylcholine on the blood vessels. Procaine curarizes first the intercostal respiratory muscles, then the diaphragm and finally the skeletal muscles. Physostigmine, dimethyl-carbamate of hydroxy-phenylbenzyl-trimethylammonium (Nu-683) or di-isopropylfluorophosphate (DFP) suppress the paralyzing effects of procaine on synapses, on postsynaptic cardiac innervation and on neuro-muscular junctions, while procaine protects against most symptoms of intoxication induced by physostigmine, Nu-683 or DFP.

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Acute and Subacute Glomerulonephritis Modified by Adrenocorticotropin.* (17808)

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From the Department of Medicine, Northwestern University Medical School.

The data compromising this preliminary report were collected for the purpose of investigating the acute or subacute phases of glomerulonephritis under the influence of adrenocorticotropin. Attention was directed less toward phenomena associated with water and electrolyte metabolism than those associated with inflammation and with azotemia. In view of the transitory character of acute glomerulonephritis in children, with or without therapy of any sort, patients were selected in whom the disease was of long duration.

The first patient, R. L., was a 12-year-old girl who had been treated for subacute glomerulonephritis and rheumatic heart disease at Children's Memorial Hospital, for 6 weeks. Gross hematuria, azotemia and hypertension had been present throughout that period.

When admitted to Passavant Hospital, the urine was claret-colored and the 24-hour output contained an average of 7 g of protein. There was a loud apical systolic murmur; the blood pressure was 152/110 and the blood urea was 54 mg/100 cc. The erythrocyte count was 3,500,000 and the hemoglobin was 6.7 g%. Since there was no evidence of edema, she was given a general diet, without salt restriction. She was started on small doses of ACTH, totalling 27 mg daily, Armour standard. Even at this dose, the blood pressure rose abruptly, and after the fourth day the medication was discontinued for 2 days. Started again, the blood pressure appeared to be favorably affected, and the total dose was raised to 50 mg daily. Notwithstanding the fluctuations of pressure, the hematuria diminished after the second day of treatment, and by the fifth day, the urine was straw-colored. Microscopic examination of the centrifuged passed specimen, several days later, showed only an occasional erythrocyte.

There has been no reccurrence of hematuria. The blood pressure, blood chemistries, body weight, urine protein in g/24 hr are given in Fig. 1, along with the system of ACTH administration. The patient was discharged from the hospital on June 28; daily injections of ACTH, 25 mg, were given to her as an outpatient for the following month.

The second patient, C. D., was a 9-year-old girl who was admitted to Passavant Hospital with a history of rheumatic heart disease and subacute glomerulonephritis of 5 months' duration. The systolic blood pressure ranged from 176 to 188, the diastolic from 90 to 110. There was a loud systolic murmur, best heard at the apex, and a marked pulsus alternans. The erythrocyte count was 2,970,000, the hemoglobin 7.7 g, the leucocyte count 7,150. The urine was grossly bloody and contained 3.4 g of protein in 24 hours. The blood urea nitrogen was 73, the non-protein nitrogen 144 mg%; the serum cholesterol was 361 mg; creatinine 3.0 mg; total protein 5.5, albumin 2.9 g%.

After four days of control studies, ACTH was administered in doses of 20 mg, every 4 hours. By the fifth day of treatment the urine was straw-colored and microscopic examination of the morning specimen showed only an occasional erythrocyte. The diastolic blood pressure averaged 22 mm lower during this interval than during the control period. Adrenocorticotropin was then discontinued for the subsequent 5 days, at the end of which the urine had again turned dark red and the centrifuged specimen was found to be packed with erythrocytes. A second course of ACTH was associated with the disappearance of red cells, as was the first. This patient was studied for 35 days, with varying doses of ACTH. Upon discharge from the hospital the blood urea nitrogen was 42 mg/100 cc,

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* This study was aided by a grant from U. S. Public Health Division of Research Grants and Fellowships.

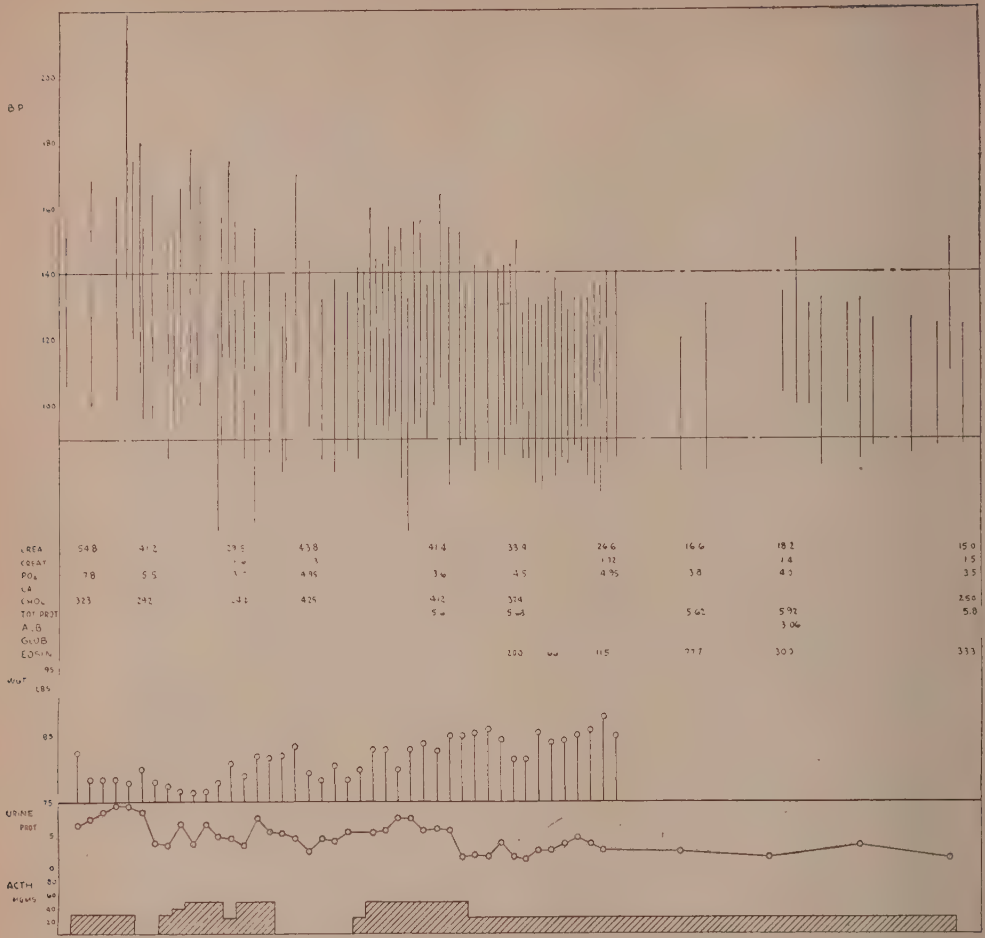

FIG. 1.
Administration of ACTH in subacute glomerulonephritis.

the creatinine 1.8; the serum cholesterol was 205 mg/100 cc. Follow-up studies 7 weeks after discharge indicate that the diastolic blood pressure remained below 90 mm. The non-protein nitrogen was found to be 44 mg/100 cc. The erythrocyte count was 4,080,000, the hemoglobin 10.5 g. Hematuria redeveloped soon after the patient returned home, and has remained present in varying degree.

The third patient was a 5-year-old girl, C. H., who was hospitalized at Children's Memorial Hospital with acute glomerulonephritis of several weeks' duration. Edema had been present on admission but had subsided soon after. The blood urea nitrogen was moderately elevated. The urine was smoky and loaded with erythrocytes. The volume was small and the concentration of protein was high. This patient was given 10 mg of ACTH every 6 hours. The eosinophils disappeared from the circulating blood, and by the fifth day the smoky urine turned straw-colored. The blood urea nitrogen came down from 28 to 15 mg/100 cc, rose to 22 mg% after 10 days of withdrawal, and returned to normal when medication was restarted. Fig. 2 shows the system of ad-

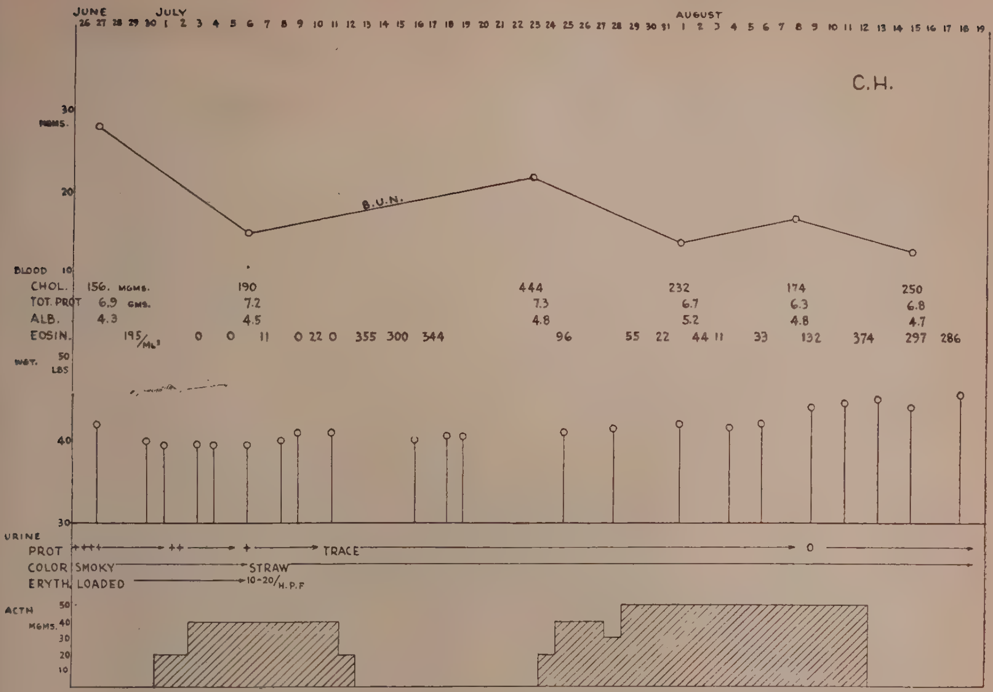

Fig. 2.
Administration of ACTH in acute glomerulonephritis.

ministration, along with the principal blood chemistries, the body weight and the urine characteristics.

Summary, results and conclusions. Two patients with subacute glomerulonephritis and one associated with rheumatic heart disease, with acute glomerulonephritis, were treated with adrenocorticotropin. In the first 2 cases hematuria was abolished or reduced to an occasional erythrocyte in the passed specimen. Azotemia and hypertension were reduced. In the second patient the improvement in blood pressure and nitrogen retention was maintained for the 7 weeks elapsing since the experimental period; however, hematuria recurred shortly after the discontinuation of treatment. In the first, follow-up studies performed 8 months after treatment showed a normal blood pressure, absence of blood in the urine but a slight increase in the blood urea and non-protein nitrogen.

Because of the relatively short duration of the disease in the third patient, less significance is attributed to the abatement after administration of ACTH.

It is concluded that hematuria, azotemia and hypertension associated with the active phases of glomerulonephritis can be favorably modified by activation of the adrenal cortex with ACTH.

Adrenocorticotropin was furnished through the courtesy of Dr. John R. Mote of Armour Laboratories.

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Metabolic Changes Associated with Administration of Adrenocorticotropin in the Nephrotic Syndrome.* (17809)

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This study was undertaken for the purpose of determining and describing the metabolic changes associated with the activation of the adrenal cortex by adrenocorticotropin in the nephrotic syndrome. Since the mechanism and duration of such activation are still obscure in the normal as well as in the disease entity reported here, efforts were made to vary the periods of administration and to prolong the periods of observation following courses.

Eight patients were treated with adrenocorticotropin. The subjects varied in age from 3 to 37 years. Six were males. In all cases the disease had been present for over 3 months. With one exception the blood urea nitrogen was found to be above the upper limits of normal in all cases, prior to administration of adrenocorticotropin. In one patient nitrogen retention was marked; the clearance of mannitol was 7 cc/min. Papilledema and retinal hemorrhages were present and the blood pressure was consistently elevated. (See Fig. 1).

Methods. The total daily dose of ACTH varied from 40 mg to 100 mg, Armour standard, given in divided doses every 6 hours. Courses of administration were also varied in duration and in 4 instances were repeated several times. All patients were maintained on dietary salt intakes of 700 mg or less, daily. In 6 cases metabolic studies included nitrogen balances, weekly blood chemistries, frequent or daily eosinophil counts, determinations of sodium, potassium, chloride, phosphorus, creatinine, total nitrogen and protein, from 24-hour urine collections. The blood chemistries were performed according to standard procedures. Sodium and potassium determinations were done with the Beckman flame photometer. Eosinophil counts were made in the hemocytometer chamber, employing the differential stain developed by Randolph.†

Results. The majority of patients manifested a more or less marked increase in edema during the administration of ACTH. In 5, the course was followed within a period which varied from a few hours to 3 days, by a diuresis which developed to the point of dehydration and was indicated by a weight-loss of 17 lb in a 7-year-old and 40 lb in a 17-year-old. One patient failed to initiate diuresis during 2 courses but after the tenth day of the third course she regained her normal weight within 4 days while still receiving the medication. The seventh patient, a 3-year-old-boy, showed no significant diuretic response to several brief courses. When diuresis was finally established, dehydration was evident in the tissues of face and limbs, but a residuum of ascitic fluid persisted. In the eighth patient, a 37-year-old man with massive ascites, repeated experimental periods resulted in a negligible reduction in edema.

In all cases, the amounts of adrenocorticotropin administered were sufficient to suppress the circulating eosinophils.

In those patients in whom nitrogen studies were possible, the balance was consistently negative during administration of ACTH and for 3 or 4 subsequent days, after which a consistent and marked positive balance appeared. During this period the serum proteins rose substantially. Improvement in serum cholesterol tended to parallel that of the serum proteins. The blood urea nitrogen decreased in all cases. Control blood sugar levels were generally low and all values showed an increase during the experimental period.

Urinary protein diminished considerably but disappeared entirely in only one patient.

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* This study was aided by a grant from the U. S. Public Health, Division of Research Grants and Fellowships.

† Randolph, T. G., Blood Studies in Allergy, I. The Direct Counting Chamber Determination of Eosinophils by Propylene Glycol Aqueous Stains, *J. Allergy*, 1949, v15, 89.

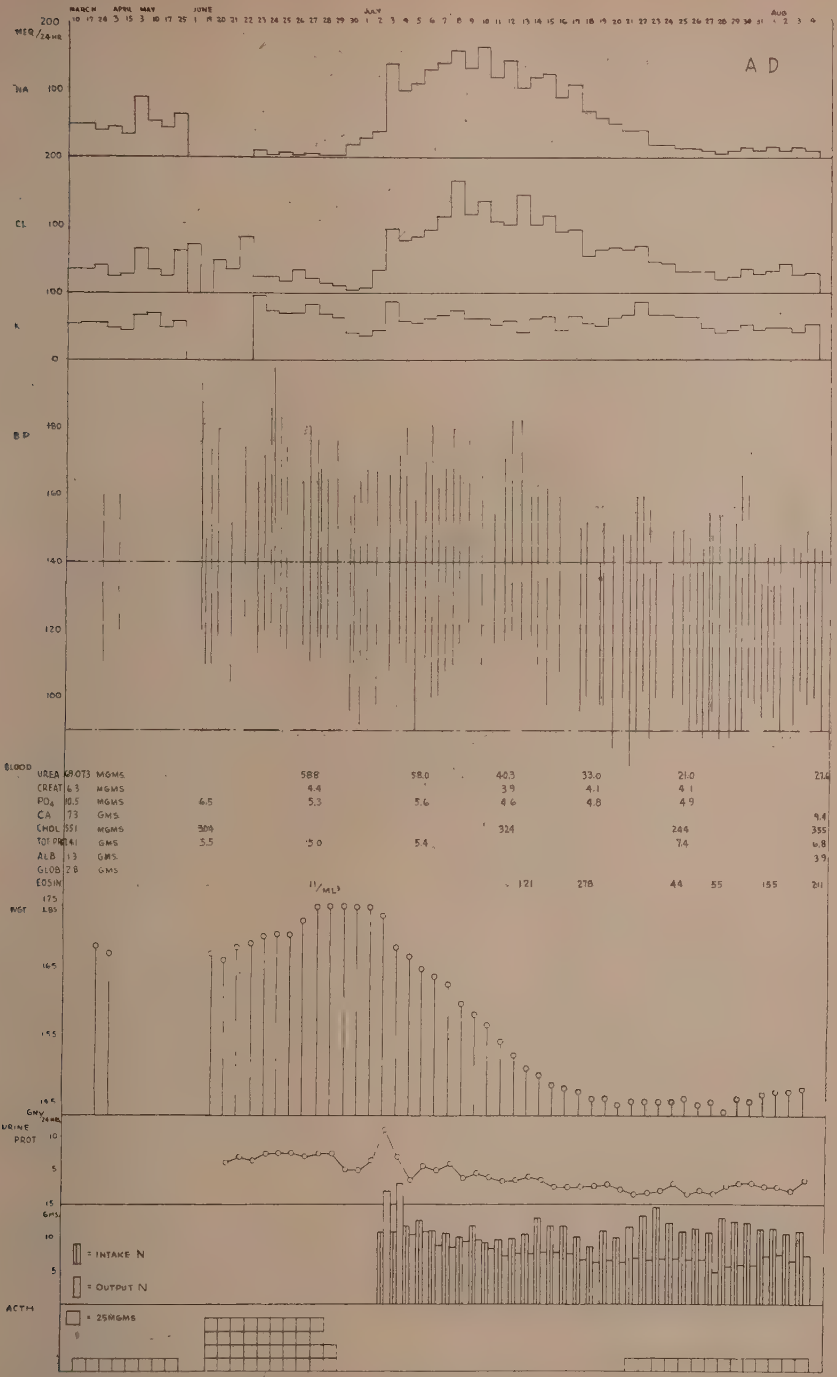

FIG. 1.

Administration of ACTH in a patient with nephrosis, azotemia, and hypertension.

Potassium output showed only inconspicuous fluctuations. In one patient the daily excretion of phosphorus was liberally increased during treatment.

Attempts were made to maintain 3 of the group by the daily administration of ACTH, 25 mg, or by weekly administration of 100 mg in divided doses. With both systems, fairly satisfactory control was secured for intervals of 6-8 weeks. Three to 4 weeks after discontinuation of medication, indications of relapse began to appear. In one instance, later repeated courses of ACTH failed to bring about diuresis, a decrease in serum cholesterol or an increase in serum proteins. The second patient has had no further treatment since Oct. 6; he shows no evidence of salt and water retention; the blood urea is at the control level. The serum cholesterol is 294 mg% and the total protein is 6.6 g% with an albumin value of 3.4 g%. Prior to treatment the total protein was 4.0 g %; the albumin 1.1 g %. The third patient discontinued treatment on the same date. He showed no tendency to redevelop edema until 3 months later, at which time the serum proteins diminished sharply.

Conclusions. It is concluded that an appropriate stimulus to the adrenal cortex by the administration of adrenocorticotropin is capable of producing marked modifications in the nephrotic syndrome. These modifications include reduction in serum cholesterol and urine protein, increase in serum proteins and profound diuresis. With one exception the diuresis occurred after discontinuance of ACTH, and the improvement in nitrogen balance and serum values continued for several weeks.

Adrenocorticotropin was furnished through the courtesy of Dr. John R. Mote of Armour Laboratories.

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Effect of Penicillin Dosage Schedule on Treatment of Experimental Typhoid Infections in Mice. (17810)

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(Introduced by L. Earle Arnow.)

From the Medical Research Division, Sharp and Dohme, Inc., Glenolden, Pa.

It has been reported by Zubrod(1) and by White and his co-workers(2) that, for the treatment of experimental streptococcal infections in mice, the total amount of penicillin required does not vary over a fairly wide range of dosage schedules. Since it is our opinion that the major problems of penicillin therapy are now centering around the management of infections caused by organisms of high penicillin resistance, we have studied the relationship between dosage schedules and the amount of this antibiotic agent required to protect mice infected with the Panama No. 58 strain of *Salmonella typhosa*. This organism has been selected because it is quite resistant to the action of penicillin in comparison with the highly susceptible streptococci used by the above mentioned workers. When *Salmonella typhosa* was used as the infecting agent, it was found that there were marked differences in the amount of penicillin required for therapy depending upon the number and frequency of the doses employed.

These data, which can be obtained with adequate precision only from animal experimentation, are being presented because it is believed that the findings have important application to the use of penicillin in the treatment of infections in human patients, where it is desired to use the antibiotic agent with maximum efficiency and economy.

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Methods and materials. Female mice of the C.F. 1 strain weighing 16-18 g, were infected intraperitoneally with 0.5 cc of 5% mucin containing 10,000 minimum lethal doses of Panama No. 58 strain of *Salmonella typhosa,* and were treated on different dosage schedules by the intramuscular injection of 0.05 cc of an aqueous solution of crystalline penicillin G. Therapy was confined to a 24-hour period, and, in all cases, the first injection was given at the time of infection (*i.e.,* at zero hour). The animals were observed for a period of 7 days, after which time the amount of penicillin required to protect 50% of the animals (PD_{50}) was calculated by the method of Reed and Muench(3).

Results. Average results from 3 experiments are shown in Fig. 1. It can be seen that in these experiments the PD_{50} was found to vary with the treatment schedule. The total amount of penicillin required to protect 50% of the infected animals when treatment was confined to a single injection at zero hour was 3300 units. When 2 doses were given, one at zero time and the second 12 hours later, individual doses of 2450 units or a total of 4900 units were required. In this phase of the work, as the number of equally spaced injections increased, the total amount of penicillin used also increased, although the size of the individual doses decreased.

Included with the above experiments, using the same preparations of challenge organism and comparable groups of mice, was a study to determine the effect of the time interval between 2 injections on the PD_{50} penicillin value. Here all the infected mice were treated with 2 equal doses of the antibiotic agent: the first injection was given at zero hour; the time of the second treatment varying from 2 to 36 hours after the first. Fig. 2 shows the average results from this phase of the three experiments.

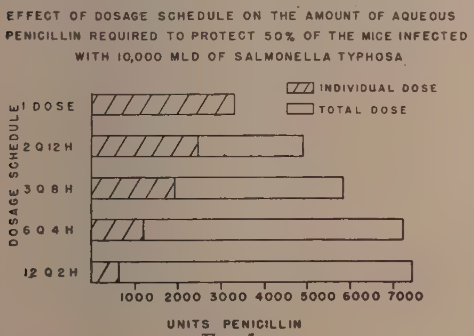

FIG. 1.

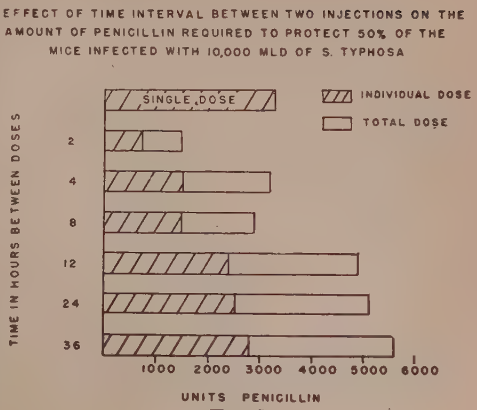

FIG. 2.

The total amount of penicillin needed to protect 50% of the mice when the 2 injections were spaced two hours apart was 1500 units, an amount approximately one-half that required for the single injection treatment. When the injections were spaced at 4- or at 8-hour intervals, the amount of penicillin required approximated the single dose schedule. Increasing the interval between injections to 12, 24, or 36 hours greatly increased the PD_{50} value.

Discussion. These data show that in the treatment of an acute typhoid infection in mice the amount of penicillin necessary to protect 50% of the animals does vary with the dosage schedule used. In a previously published paper(4), it was suggested that the intensity of penicillin therapy may be represented as a function of the relationship between the concentration of penicillin and the time during which this antibiotic agent remains at or above a minimal antibacterial

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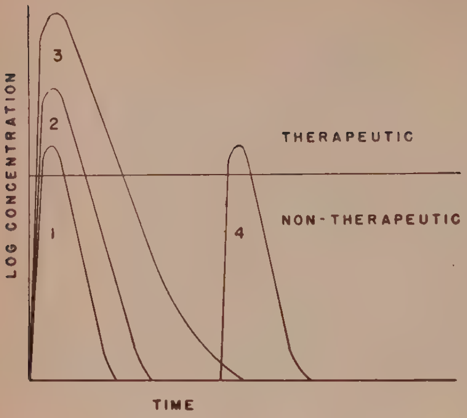

Fig. 3.

concentration in the body. If this concept is visualized in terms of a curve in which penicillin concentration is plotted against time, then intensive therapy is represented by that area of the curve that is above a minimal baseline. The position of this baseline on the ordinate is dependent on the sensitivity of the infecting organism. When the baseline is high, as for a resistant organism, and the size of each injected dose is small, then a proportionally large amount of each dose contributes only to the area below the baseline which does not represent intensive therapy. This is illustrated by curve 1 of Fig. 3. As the unitage of the individual dose is increased, the proportional amount of the injected penicillin that contributes to the effective area progressively increases as in curves 2 and 3. This would result in more efficient therapy, and would explain the corresponding decrease in the total amount of penicillin that was required in our experiments as the number of equally spaced injected doses decreased from 12 to 1 and the size of the individual doses increased from 620 units to 3300 units.

For therapy to be confined to a single injection, however, (*e.g.*, curve 3) it is necessary that this one dose of penicillin be sufficient to destroy most or all of the invading organisms. If the size of this dose is too small (*e.g.*, curve 1) the concentration of penicillin that diffuses into the area of infection may be so low that some of the infecting organisms may be injured but not destroyed. Some cells, indeed, may escape completely any deleterious effect of the antibiotic agent. In the absence of further therapy, these survivors later may multiply and re-establish the infection, leading to the death of the animal. When a second small injection closely follows the initial dose (curve 4, following curve 1), the residual organisms may be eliminated by the resulting augmentation of the killing effect of the antibiotic agent. As the interval between the two treatments is lengthened beyond the period of recovery for the injured and surviving organisms, the size of the individual effective dose gradually increases until each dose approaches the concentration required to protect with a single dose.

Summary and conclusions. When mice were infected with a relatively penicillin-resistant *Salmonella typhosa,* it was found that the penicillin PD_{50} varied with the treatment schedule. A smaller total amount of penicillin was required for protection when the treatment was given as a single injection than when 2, 3, 6, or 12 doses were divided equally within a 24-hour period. When the time interval between 2 doses was varied from 2 to 36 hours, it was found that the PD_{50} for the 2 dose 2-hour schedule represented an even smaller total amount, and, therefore, a more economical use of penicillin than did the single injection schedule.

These data would suggest that in the treatment of infections caused by organisms of high penicillin resistance, the common practice of dividing the total daily amount of penicillin into frequently injected doses may be an inefficient means of therapy. It would appear that the total amount of penicillin to be used in one day would be more effective if given as a few large doses. This procedure would result in the higher plasma penicillin concentrations and the correspondingly increased tissue concentrations required for a bacteriocidal action against relatively resistant organisms.

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Prevention of Loss of Body Fat by Cortisone. (17811)

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From the Merck Institute for Therapeutic Research, Rahway, N. J.

Fragmentary evidence for an unclarified action of the adrenal cortex upon fat metabolism has appeared in the literature. The subject has been reviewed by several authors (1,2,3). Recently, Schiffer and Wertheimer (4) reported that total body fat was much lower in adrenalectomized rats than in "paired fed" controls. In their experiments the excessive loss of fat was prevented by the administration of desoxycorticosterone acetate (DCA) but not by whole adrenal cortical extract.

In the present experiments, it was observed that in the fully fed animal, adrenal insufficiency had no influence upon the amount of body fat. In contrast, partially starved, adrenalectomized rats lost fat more rapidly than intact animals on the same caloric intake. The excessive loss of fat in adrenalectomized rats on a restricted food intake was prevented by the administration of cortisone acetate (17-hydroxy-11-dehydrocorticosterone acetate) but not by DCA.

Experiments. Male rats of the Sprague-Dawley strain weighing from 165-230 g were grouped as indicated in the tables. Until the start of the experiments the animals had access to a complete stock diet and to water. During the 5-day period after operation the rats (Group I-VII in Table I) were fed 3 g of diet in the morning and 3 g in the evening. This quantity, representing about one-third of the rats' food intake when fed *ad libitum*, was consumed completely by the animals of all groups. Groups VIII to XI (Table II) were maintained on the restricted food intake for 8 days. The animals were then operated upon and thereafter permitted food *ad libitum*. Adrenalectomies were performed under ether anesthesia by the lumbar approach. Intact rats had continuous access to tap water, adrenalectomized animals were given 1% NaCl. The cortisone acetate employed was Cortone Merck which is a saline suspension of the micronized material. Desoxycorticosterone acetate was similarly suspended before administration. One mg of cortisone acetate was administered subcutaneously twice daily to the animals in groups VII and XI, while the rats in group VI received two doses of 1 mg of DCA per day. After 5 days of treatment, the animals were killed with ether and each liver and testicular fat body was dissected and weighed. In groups III, IV, VI and VII, the fat tissue was returned to the decapitated carcass which was minced in a meat grinder after removal of the intestines. Total lipids were extracted with alcohol from the ground carcasses and livers(5). For these extractions the pooled carcasses and livers from 4 animals of each of the groups examined were used. Cholesterol, free and total, was determined according to Sheffel(6). Total phosphorus was determined by the method of Fiske and SubbaRow(7) and the factor 25 was used for the conversion to phospholipid values(5).

Results. Previous experience of others(8) confirmed by our own has demonstrated that the testicular fat body is a structure with as highly a reproducible weight and relative growth(9) as that of any other organ. The average weight of the testicular fat body in Groups I and II was used as a base line for the calculated loss of fat in the animals in Groups III-VII. A comparison of the loss of adipose tissue in the various groups showed

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TABLE I.
Effect of Adrenal Activity on Loss of Weight of Testicular Fat Body. All operated rats on restricted food intake for 5 days.

		G			G % of final body wt		
Experiment	Group	Initial body wt	Final body wt	Body wt loss	Wt of fat body	Loss of fat	No. of rats
Intact controls	I	—	200	—	.480	—	12
” ”	II	—	175	—	.436	—	10
Sham op. contr.	III	200	181	19	.395	.085	16
Adrx.	IV	195	182	13	.287	.193	8
”	V	176	157	19	.243	.192	8
” + DCA	VI	175	162	13	.266	.169	8
” + KE	VII	200	167	33	.436	.054	8

TABLE II.
Effect of Adrenal Activity on Weight Gain of Testicular Fat Body. All rats on restricted food intake for 8 days, then operated and fed *ad libitum* for 7 days.

		G			G % of final body wt		
Experiment	Group	Initial body wt	Final body wt	Body wt gain	Wt of fat body	Gain of fat	No. of rats
Intact controls	VIII	—	148	0	.149	0	5
Sham op. contr.	IX	146	203	57	.318	.169	6
Adrx.	X	144	189	45	.287	.138	6
” + KE	XI	142	154	12	.360	.211	6

that sham operated rats (Group III) lost less than one-half of the amount of adipose tissue lost by adrenalectomized animals (Groups IV and V) on an isocaloric food intake. The loss of fat in underfed, adrenalectomized rats (Groups IV and V) was about 4 times greater than that in similar animals receiving 2 mg of cortisone acetate daily (Group VII), in spite of the fact that in these rats, body weight loss was much greater than in any of the other groups. The administration of 2 mg of DCA (Group VI) had no significant retarding effect upon the rate of disappearance of the adipose tissue.

The neutral-fat, phospholipid, and cholesterol contents of carcass and of liver are compared in Fig. 1. These values were determined in Groups III, IV, VI and VII. The changes in the amount of neutral-fat in the carcasses as well as in the livers, closely paralleled the changes in the weights of the testicular fat bodies. Phospholipid and total cholesterol concentrations were identical in the carcasses of all 4 groups. However, liver phospholipid concentrations appeared to be altered in the same direction as the neutral-fat. Perhaps worthy of further mention is the apparently marked increase in the ratio of cholesterol ester: total cholesterol in the carcasses, but not in the livers, of animals injected with cortisone acetate (Group VII) and the relative decrease of phospholipids in the livers of the same animals.

The recovery of the fat body in animals first starved, then adrenalectomized, and then fed *ad libitum* (Group X) was essentially the same as in the sham operated controls (Group IX). Adrenalectomized animals injected with 2 mg of cortisone acetate daily regained much less body weight but gained somewhat more adipose tissue than any of the other groups.

Discussion. The apparent discrepancy between our findings and those of Schiffer and Wertheimer appears readily explained from the following: for reasons not given by the authors, in Schiffer and Wertheimer's experiments adrenalectomized rats were exposed to the stress of a relatively high K^+ intake. In addition to a diet apparently quite high in this element, 0.035% of KCl was given with 0.7% NaCl and other salts in the drinking water. It is clear that their adrenalectomized rats, although they had access to food *ad libitum,* failed to eat and gain weight when not given DCA, but ate and grew normally when injected with DCA, which counteracts the increased toxicity of K^+ in

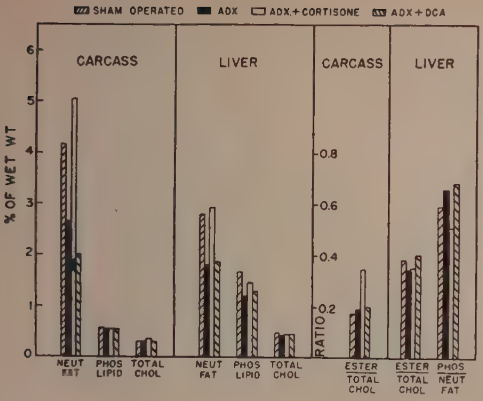

Fig. 1.

Comparison of neutral-fat, phospholipid, and cholesterol contents of carcass and of livers in sham operated (Group III), adrenalectomized (Group IV), adrenalectomized + cortisone (Group VII), and adrenalectomized + DCA (Group VI) rats.

adrenalectomized animals(10). The action of DCA in these experiments appears to be primarily upon the restoration of appetite in adrenalectomized rats exposed to the stress of a relatively high K^+ intake. "Paired fed" intact animals were used by Schiffer and Wertheimer to control the effect of adrenal insufficiency (complicated by high K^+ feeding) but not paired fed adrenalectomized controls injected with DCA. In the present experiments, caloric intake was controlled and was the same for all partially starved groups. Under these conditions it was seen that cortisone acetate prevented the excessive loss of fat in partially starved, adrenalectomized rats while DCA had no such effect. Furthermore, in adrenalectomized rats fed *ad libitum*, no appreciable abnormality could be detected with respect to the amount of body fat. Animals starved prior to adrenalectomy and then permitted free access to food regained adipose tissue at essentially the same rate as sham operated controls. However, in adrenalectomized rats receiving cortisone acetate the gain of fat tissue, in spite of a markedly retarded recovery of the body weight, was even somewhat greater than in the other groups. Although from the above it appears justified to question the validity of some of Schiffer and Wertheimer's conclusions, it is obvious that their experiments have yielded important information. It was seen from their work that the deposition of labelled fat was unimpaired in the underfed adrenalectomized rat and that the excessive loss of fat in such animals occurs irrespective of whether the rats were consuming a diet high in protein, carbohydrate or fat. Also, no differences in the amounts of fecal fat were demonstrable.

From these findings and from the present observations, it is evident that adrenal cortical activity influences both fat and carbohydrate in a parallel fashion. Since partially starved, adrenalectomized rats utilize more fat than cortisone injected controls, it is logical to assume that the deficit in carbohydrate is likewise due to excessive utilization. The metabolic changes in adrenal insufficiency, rather than being primarily due to reduced protein catabolism and decreased gluconeogenesis, may then perhaps be better explained as a result of decreased utilization of amino acids which causes a compensatory excessive utilization of carbohydrate and of fat. Conversely, overdosage of cortisone would lead to increased utilization of amino acids followed by sparing of carbohydrate and of fat.

Summary and conclusions. Adrenalectomized rats, partially starved for 5 days, lost approximately 4 times as much adipose tissue as similar animals injected with 2 mg of compound E daily and twice as much as sham operated controls. The daily injection of 2 mg of DCA did not prevent the excessive loss of fat in adrenalectomized animals. The weight changes of the adipose tissue were paralleled by changes in the content of neutral-fat in the carcasses and livers of the same animals. No appreciable lag in recovery of adipose tissue was observed when starved rats were adrenalectomized and then fed *ad libitum*.

We wish to thank Miss Gertrude Ellenberger for valuable assistance.

Received March 17, 1950. P.S.E.B.M., 1950, v74.

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Agglutination of Cercariae of *Schistosoma mansoni* by Immune Sera.* (17812)

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Although there are several immunologic tests which reflect helminthic infection(1), there is considerable need for an adequate and accurate test which will help one to measure the response of an infected host to the invading parasite. We here report on the presence of an agglutinin for the cercariae of *Schistosoma mansoni* which develops in the blood of monkeys. The agglutinin appears early in the course of the infection not only with the *S. mansoni* but is also found in monkeys recently infected with Trichinella. We have studied some of the characteristics of the reaction and tentatively classify it as an antigen antibody reaction.

Materials and methods. Cercariae were obtained from infected snails, *Australorbis glabratus*,† by placing them under a bright light for 2-3 hours in a beaker. A concentrated cercarial suspension was obtained by centrifuging the original suspension at about 1000 RPM for one minute. An equal volume of 1.7% sodium chloride solution was added slowly thus yielding a final suspension of cercariae in 0.85% saline. Usually a suspension of 300-400 cercariae per ml was used for the test. Sera were inactivated by heating in a water bath at 56°C for 30 minutes. Serial dilutions were made in 0.85% saline and 0.5 ml of cercarial suspension was added to 0.5 ml of the diluted serum. The test tubes (10 x 100 mm) were shaken vigorously for about 30 seconds and then left at room temperature for one hour. Agglutination usually occurred within the first half hour but the final reading was made after 4°C for 18 hours. The agglutination was read as one to 4+ positive. A 1+ agglutination was determined with the aid of a microscope. The titer was reported as the final dilution of serum in the tube with 1+ agglutination. The production of agglutinins was followed in two rhesus monkeys which were given 900 and 600 *S. mansoni* cercariae by intraperitoneal injection. Blood was drawn by cardiac puncture at weekly intervals. The direct enumeration of circulating eosinophiles (2), a total leucocyte and differential count was made at each bleeding. Serum was obtained from centrifuged clotted blood. Stool examinations by the 10% ethyl alcohol sedimentation method(3) were started near the end of the incubation period.

Results. Formation of the Agglutinate. The agglutination of cercariae was first noticed when a large number of cercariae were placed in a serum and then observed under a cover slip for the formation of a precipitate around the individual cercariae(4). This serum had been obtained from a monkey which had had *S. mansoni* for 2 years. It was then noticed that the cercariae stuck to one another whenever they came into contact so that eventually masses of entwined cercariae were formed. (Fig. 1) This did not occur in normal sera. (Fig. 2) Following this, it was decided to study the phenomenon in the test tube with varying dilutions of sera and the technique outlined above was evolved. The clumping sometimes occurred in tubes within 2 to 3 minutes but usually took place in 20 to 30 minutes at room temperature. There appeared to be no regular pattern in which one part of the

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* Supported in part by a contract with the Veterans Administration.

1. Culbertson, J. T., *Columbia University Press*, 1941.

† Courtesy of Dr. E. B. Cram, Division of Tropical Diseases. National Institutes of Health, Bethesda, Md.

2. Forsham, P. H., Thorn, G. W., Prunty, T. T. G., and Hills, A. G., *J. Clin. Endocrin.*, 1948, v8, 15.

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4. Papirmeister, B., and Bang, F. B., *Am. J. Hyg.*, 1948, v48, 74.

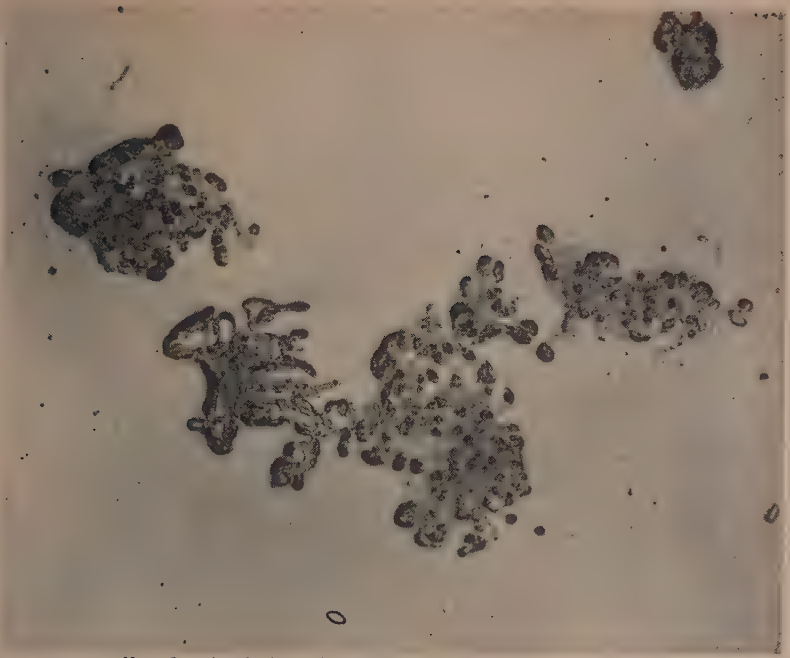

FIG. 1. Agglutinated cercariae in immune serum. ×25.

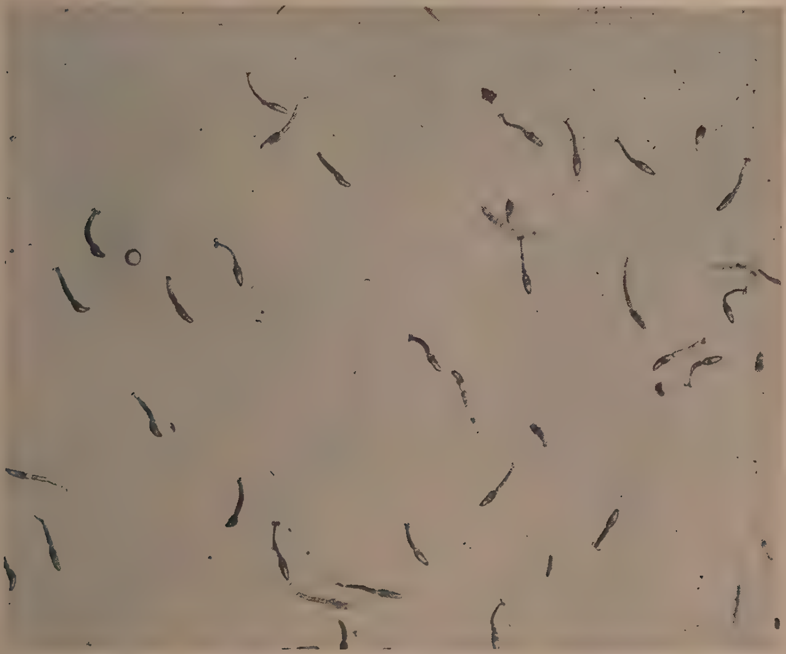

FIG. 2. Cercariae in normal serum. Not agglutinated. ×25. Serum and cercariae were mixed in a test tube for 30 minutes at room temperature and the combination was transferred to a slide for photographing.

TABLE I.
Demonstration of Prozone Phenomenon in Immune Schistosome Monkey Sera on Agglutination of Cercariae.

	Dilution						
Monkey No.	1/2	1/4	1/8	1/16	1/32	1/64	1/128
5	0	0	0	++++	++++	++++	+++
29	+	+	+	++	++	++	+
14	0	+	+	++++	++++	++++	+++

TABLE II.
Agglutinin Titer on Monkey Infected with *S. mansoni* March 8, '48.

	Dilution					
Bled	1/4	1/8	1/16	1/32	1/64	1/128
4/20/48	+	+++	++++	++++	0	0
6/14/48	0	0	+	++++	++++	±
7/26/48	not done		+++	+	+	0
10/15/49	+	+	++++	++++	+	0

cercariae stuck to another, and the motility of the cercariae was still present after 24-48 hours at 4°C. Up to date, only living cercariae have given the agglutination. Cercariae killed either by heat, formalin, ultraviolet light or chemicals have not given agglutination. The age of the cercariae has had very little effect on the agglutinin titer. Positive agglutination had been obtained even in a 24-hour-old living cercarial suspension, and washing of living cercariae with 0.85% saline did not affect the agglutination. Finally a prozone phenomenon was consistently present in several sera as shown in Table I.

Development of agglutinins in experimentally infected monkeys. The time of appearance of agglutinins was studied by infecting 2 monkeys with 900 and 600 cercariae of *S. mansoni.* The appearance of agglutinins which was roughly concomitant with the appearance of eggs in the stools is shown in Fig. 3 and 4. That the antigen stimulating this antibody response is not unique to the schistosome worms is indicated by a study of the serum of a monkey infected with trichinella. This monkey also developed agglutinins for schistosome cercariae on the 34th day after having been fed some 620 trichina larvae. The titer increased to 1/64 on the 50th day at which time the monkey was challenged with 50-60 *S. mansoni* cercariae cutaneously. The antibody titer rose to 1/128 on the 58th day and stayed approximately at this level thereafter. There was a marked but temporary eosinophilia up to 2200/cmm accompanying the initial rise of the agglutinin. This monkey died on the 43rd day of schistosome infection due to rupture of the heart from cardiac puncture.

Autopsy showed 6 pairs of matured schistosomes in the mesenteric veins leading from the large intestines. Many yellowish-white nodules were found on the surface of the liver and in the mucosa of the large intestines. No ulcerations were present. These nodules contained living schistosome eggs when examined by pressing between two slides. Examination of the diaphragmatic muscle by a similar compression method(5) showed a moderate number of encapsulated trichina larvae. Thus, there is no evidence of protection produced by one infection against the other. The agglutinins apparently remain present in high titer for at least a year and a half as shown by a study of 2 monkeys (*M. irus*) which had been previously infected. In one the serum drawn 6 weeks after infection and stored at about –16°C was found to have a titer of 1/32. This titer later went to 1/64 and was maintained throughout as shown in Table II.

We cannot, however, conclude that the

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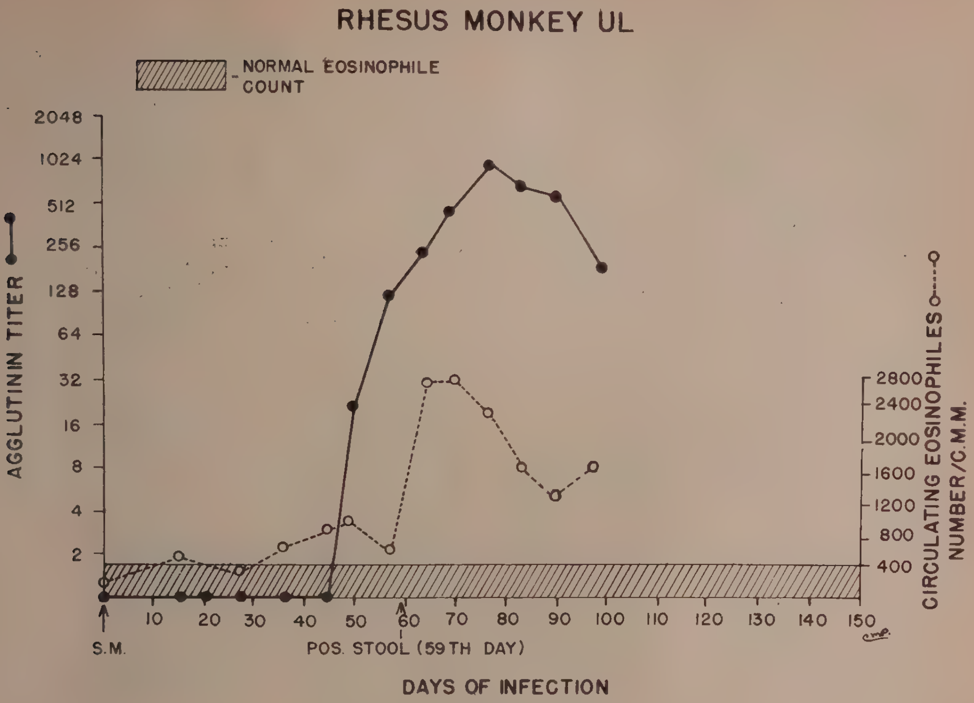
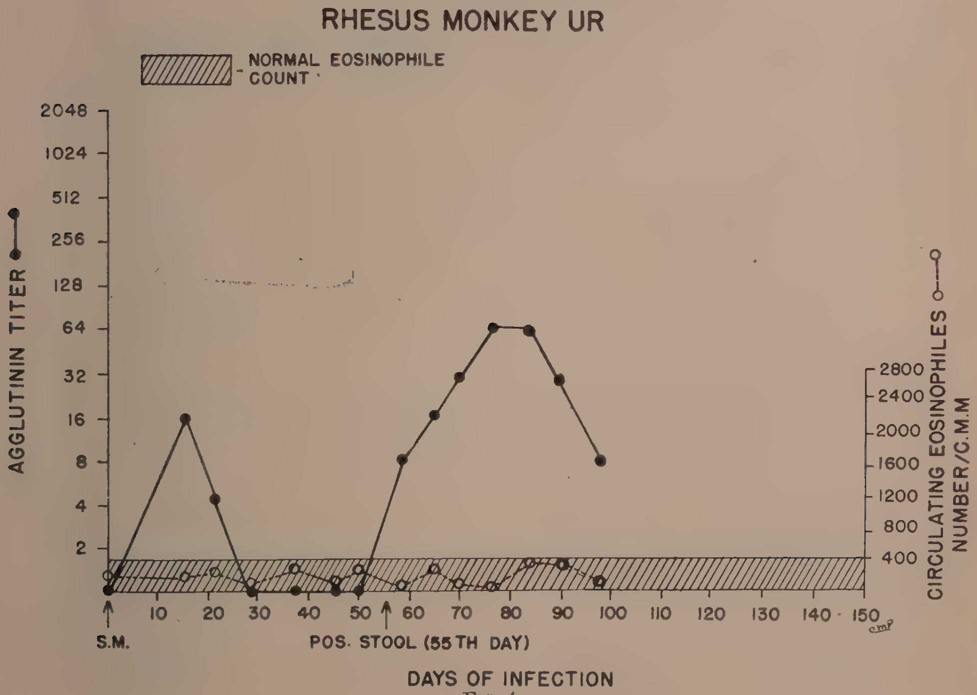

RHESUS MONKEY UL
▨ - NORMAL EOSINOPHILE COUNT
AGGLUTININ TITER ●—●
2048
1024
512
256
128
64
32
16
8
4
2
CIRCULATING EOSINOPHILES ○---○
NUMBER/C.M.M.
2800
2400
2000
1600
1200
800
400
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
S.M.
POS. STOOL (59TH DAY)
DAYS OF INFECTION
cmp.

FIG. 3.


RHESUS MONKEY UR
▨ - NORMAL EOSINOPHILE COUNT
AGGLUTININ TITER ●—●
2048
1024
512
256
128
64
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16
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4
2
CIRCULATING EOSINOPHILES ○—○
NUMBER/C.M.M
2800
2400
2000
1600
1200
800
400
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
S.M.
POS. STOOL (55TH DAY)
DAYS OF INFECTION
cmp

FIG. 4.

agglutinin remains present for the duration of infection. Of 7 monkeys (*M. philippinensis*) infected with *S. japonicum* 4½ years previously, which still had active infections as demonstrated by positive stool examinations, only 3 showed agglutinins in a titer of 1/128-1/160. The others were negative. It is, of course, possible that the different species of parasites (*S. japonicum* instead of *S. mansoni*) is responsible for these negative tests, but in view of the overlap of the reaction with the trichina infection this is unlikely as an explanation. Six normal monkeys have given negative results.

Discussion. The agglutinin reaction here reported differs from the formation of a precipitate around the cercariae of *Schistosoma mansoni* in that a heat labile factor, possibly complement is necessary for the precipitate reaction(4). It may, however, have some basic similarity to the reaction reported by Vogel and Minning(6) in which a sharply defined homogeneous membrane is formed around the individual cercariae when put in either fresh or inactivated sera. We, however, have not observed any membrane formation during our tests for agglutination. The cross reaction between trichinosis and schistosomiasis was not unexpected for we had previously found that fresh sera from monkeys infected with trichina would form a precipitate around the cercariae of *S. mansoni*(7).

The application of this test to studies in man remains to be explored. Of some seven sera from old but active infections in man with *S. mansoni*‡ or *S. japonicum*, only one *S. mansoni* serum was positive in a dilution of ⅛. We have not tested any sera from humans known to be recently infected.

Summary. The agglutination of living cercariae of *Schistosoma mansoni* by immune sera in high dilutions is reported. This reaction which occurs in heat inactivated as well as in fresh serum also appears in the serum of monkeys recently infected with trichina. The agglutinin develops early in the acute infections and was absent in four of seven chronic schistosome infections.

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7. Bang, F. B., and Papirmeister, B., unpublished data.

‡ Courtesy of Dr. H. Most, New York University Medical College, N. Y. City.

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Practical Application of a Hemagglutination Reaction in Tuberculosis.* (17813)

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Serological methods in the past have been unsatisfactory in the diagnosis of tuberculosis. Although a variety of immunological techniques had been employed, consistent results from different laboratories were not obtained. Sera from many patients with active tuberculosis failed to react, and in turn, the sera from many normal individuals showed positive tests by these techniques. Difficulties may have arisen from the lack of a suitable antigen of known chemical composition which was capable of reacting with its corresponding antibody. These difficulties were for the most part obviated in 1948 when Middlebrook and Dubos(1) described a specific hemagglutination reaction between sheep erythrocytes treated with a water soluble fraction (rich in polysaccharide) of mammalian tubercle bacilli and the sera of tuber-

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* This study was supported by a joint grant-in-aid from United States Public Health Service and the National Tuberculosis Association.

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culous patients. Because of the difficulty in preparing large quantities of the polysaccharide antigen, it became desirable to find a more easily available supply of antigenic material for a large scale application of this reaction. The studies of earlier workers(2,3) suggested the applicability of old tuberculin as an antigen in tuberculosis serological reactions. One preparation of old tuberculin, Gilliland O. T. (Wyeth), was found by Middlebrook and Dubos(1) to be effective in sensitizing sheep erythrocytes. Scott and Smith(4) have also recently reported the use of another preparation, old tuberculin (Lederle), as a suitable antigen for the hemagglutination test. Of several old tuberculins tested, we noted old tuberculin concentrated approximately 4 times its normal strength, prepared by Lederle Laboratories, to be an excellent antigenic substance and it was therefore used in the studies herein reported.

Materials and methods. The preparation of the materials and the technique of the hemagglutination test were the same as described by Middlebrook and Dubos(1) with the exception of the following points: (a) Instead of the polysaccharide extract of tubercle bacilli, the antigen used for sensitization of the sheep erythrocytes consisted of concentrated old tuberculin (Lederle)[†] diluted 1:12 in physiological saline with pH adjusted to 6.8-7.0 by addition of dilute NaOH. (b) In the absorption of serum with untreated sheep red cells to remove antibodies unrelated to tuberculous infection, it was found that a single absorption of 2 cc of 1:2 dilution of inactivated serum with 0.2 cc of packed, washed, untreated sheep red cells in a water bath at 37°C for 30 minutes was sufficient. (c) Hyperimmune rabbit serum was employed as a positive control for the antigen-sensitized sheep cells. This serum was prepared by the intravenous injection of 1 cc of heat-killed bovine tubercle bacilli (Ravenel) biweekly for 4 weeks. The bacilli were grown for 10 days in Tween-albumin media(5,6) at 37°C, centrifuged, resuspended in saline and heat-killed at 60°C for 45 minutes. The rabbit serum was inactivated and absorbed in the same manner as the patients' test sera. The human sera to be tested were obtained from in-patients, out-patients, and donors to the blood bank of the Montefiore Hospital and the Montefiore Country Sanatorium. Additional sera were supplied through the kindness of physicians on the staff of the Division of Pulmonary Diseases of this hospital. Sera from patients with syphilis all gave strongly positive Wassermann reactions and were made available by Dr. Widelock of the Serological Laboratories of the New York City Department of Health. As far as could be ascertained skin tests with old tuberculin or any of its products were not performed on patients with active pulmonary tuberculosis.

Results. Results of the hemagglutination tests for tuberculous antibodies in the sera of normal adults, of patients with syphilis, and of patients with non-tuberculous diseases of the chest are summarized in Table I. The sera from patients with syphilis were included because in the past such sera often gave heterologous reactions with the complement fixation test in tuberculosis. Of sera from 110 normal adults, 105 contained no antibodies, 4 had titers of 1:2 and one a titer of 1:4. From the sera of 106 patients with non-tuberculous diseases, 98 gave a negative reaction and 8 showed reactions in serum dilution of 1:2. Thus in this control series of 216 sera, there were 203 with no reactions; 12 with a reaction of 1:2; and one with a reaction in 1:4 dilution of the serum.

In contrast, the results of testing the sera of patients with active tuberculosis, presented

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3. Muether, R. O., and Macdonald, W. C., *J. Lab. and Clin. Med.*, 1945, v30, 411.

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[†] Made available through the courtesy of Dr. Henry Piersma of Lederle Laboratories, Pearl River, N. Y.

5. Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, v83, 409.

6. Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.*, 1947, v56, 334.

TABLE I.
Hemagglutination Tests of Sera from Normal Adults and Patients with Non-tuberculous Diseases.

Diagnosis	No.	Antibody titer		
		No reaction	1:2	1:4
Normal adults	110	105	4	1
Syphilis	25	23	2	—
Bronchiogenic carcinoma	15	15	—	—
Metastatic lung cancer	15	14	1	—
Bronchiectasis	15	14	1	—
Lung abscess	10	10	—	—
Primary atypical pneumonia	8	8	—	—
Boeck's sarcoid	8	6	2	—
Malignant lymphoma	8	6	2	—
Pneumoconiosis	2	2	—	—
Totals	216	203	12	1

TABLE II.
Hemagglutination Tests of Sera from 201 Patients with Active or Apparently Cured Tuberculosis.

Lesion	No. of patients	Positive		Antibody titer		
		No.	%	Lowest	Average	Highest
Pulmonary: Minimal	32	30	93.7	1:8	1:16	1:64
” Moderately advanced	68	60	88.2	1:8	1:32	1:512
” Far advanced	60	57	95.0	1:8	1:32	1:256
Renal or bone	8	8	100.0	1:8	1:64	1:256
Total with active disease	168	155	92.3			
Pulmonary: Apparently cured	33	2		1:2	1:2	1:2

in Table II, demonstrated a high degree of activity. Of 168 patients with active tuberculosis, indicated by the demonstration of tubercle bacilli on staining or on positive culture from the sputa, gastric contents, urine or biopsy specimen, 155 or 92.3% showed positive reactions in titers of 1:8 to 1:512. It is noteworthy that some negative reactions occurred with sera from patients with minimal, moderately, and far advanced pulmonary tuberculosis. In Table II are also presented the results of tests of sera from 33 individuals who have been in good health for at least the past 7 years, but at one time did have active pulmonary tuberculosis. Two sera from this group showed reactions in 1:2 dilution, whereas 31 revealed no reactions. This observation suggests that the presence of these antibodies is in some way related to the activity of the disease.

From a group of 120 tuberculous patients whose sera have been tested in series at monthly intervals, it has been noted that in 10 the antibodies are no longer detectable by this technique. Their disappearance has been correlated with absence of disease activity and failure to demonstrate tubercle bacilli in the sputa or gastric contents on smear or culture. Additional evidence is thus provided that there is a relationship between disease activity and significantly positive reactions in this test. Details of this phase of the study will be described in a subsequent report.

Comment. The data presented confirm and extend the observations of Middlebrook and Dubos(1) on the specific agglutination of sheep erythrocytes sensitized with antigen from tubercle bacilli by sera of tuberculous patients. Other workers(4,7,8) have also recently reported successful use of this method in serological studies of tuberculosis. This hemagglutination reaction has become more

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8. Sohier, R., *Ann. Inst. Pasteur*, 1950, v78, 283.

practical with the demonstration that concentrated old tuberculin (Lederle) can be substituted as the antigen for sensitization of sheep erythrocytes instead of the polysaccharide fraction previously described(1). The heat stable polysaccharide fraction isolated from mammalian tubercle bacilli is undoubtedly contained in the old tuberculin preparation. Heterologous reactions were noted in approximately 6% of sera from non-tuberculous patients or normal adults in the present series, but these reactions are of lower titer than those obtained from patients with active tuberculosis. Because of the cross reactions, it appears probable that only titers of 1:8 or higher are indicative of tuberculous infection. The potential value of the test is suggested by the fact that 92% of tuberculous patients' sera showed positive reactions in titers of 1:8 or higher, whereas in a precipitin test for carbohydrate antibodies in human tuberculosis recently reported, 50% positive reactions were found(9).

From these observations it seems that this hemagglutination test apparently not only provides a valuable diagnostic tool, but unlike the tuberculin skin test, appears to indicate the presence of tuberculous activity and therein lies its greatest value.

Summary. The sera of 216 normal adults and patients with syphilis or non-tuberculous chest diseases were tested for tuberculous antibodies by the hemagglutination reaction: 203 showed no reactions; 12 had positive reactions in serum dilution of 1:2; and one in dilution of 1:4. The sera of 168 patients with active tuberculosis showed 92% positive reactions in dilutions ranging from 1:8 to 1:512. Sera from 33 apparently cured tuberculous individuals revealed no reactions in 31, and positive reactions in 2 in a serum dilution of 1:2. This hemagglutination reaction is apparently a relatively specific test for active tuberculosis.

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Separation of Pertussal Toxin.* (17814)

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Toxic extracts have been prepared from *Hemophilus pertussis* by a variety of methods(1-9). Pertussal toxin is lethal for mice, guinea pigs and rabbits and is dermonecrotic. It is heat-labile, easily detoxified by formaldehyde, and antigenic. Rabbit pertussal antitoxic sera will neutralize the toxin in multiple proportions and induce passive immunity in guinea pigs(10) and in rabbits and mice(11). The purpose of this investigation is to report methods for the extraction and separation of pertussal toxin from a variant toxin-producing strain of *H. pertussis*. The toxin can be extracted from dried *H. per-*

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* Aided by a grant from Lederle Laboratories Division, American Cyanamid Co.

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9. Burrell, J. I., Robbins, K. C., and Pillemer, L., *Science*, 1948, v108, 311.

10. Evans, D. G., *J. Path. and Bact.*, 1940, v51, 49.

11. Ospeck, A. G., and Roberts, M. E., *J. Infect. Dis.*, 1944, v74, 22.

tussis with 0.05 M $CaCl_2$ and can be separated from this extract by fractionation with methanol-water mixtures under controlled conditions of pH, ionic strength and temperature.

Materials and methods. (a) Preparation of organisms. H. pertussis, strain No. 29[†], a variant phase I strain, was grown in peptone broth for 7 days at 35-37°C(8,12,13). The organisms were harvested in a Sharples centrifuge, suspended in distilled water and dried from the frozen state. 0.08% suspensions of these organisms gave turbidities in a Klett-Summerson photoelectric colorimeter equivalent to 10 billion organisms per ml (13).

(b) *Determination of LD_{50}.* The dilutions of toxin were made in 0.9% NaCl containing 1.0% peptone (Bacto). 0.5 ml volumes were injected intraperitoneally into white female mice (Carworth CF No. 1) weighing 12-15 g. Three to 4 dilutions were made per sample; 6 to 8 mice were used per dilution. The LD_{50} value for the toxin was calculated after 96 hours using the method of Reed and Muench(14).

(c) *Chemical and physical methods.* All fractionation procedures were carried out in constant temperature baths. A refrigerated centrifuge (International, PR-I) was used for centrifugation. Nitrogen was determined by the micro-Kjeldahl method of Pregl and phosphorus was determined by the method described by Gomori(15). Hydrogen ion determinations were made on the glass electrode; solutions containing methanol were diluted to less than 5% methanol before pH determination. Ionic strength was determined by calculation. The ultrasonic experiments were carried out with a 9 KC Raytheon Corporation sonic oscillator Type R-22-3 at 0°C. Electrophoretic analyses were carried out in pH 8.6 veronal buffer, ionic strength of 0.10, using the Tiselius apparatus with the Longsworth modification of the optical system(16).

Results. (a) Extraction of organisms with water. A 2.5% suspension of organisms in distilled water (approximately 310 billion organisms per ml) was prepared using a Waring blendor for 15 seconds at 0°C. This suspension was stirred for one-half hour at 0°C and allowed to remain in the 0°C bath for 2 hours. The insoluble residue was removed by centrifugation at 4000 r.p.m. for one hour at 0°C and the supernatant solution recentrifuged under the same conditions. This extract contained 360 LD_{50} per ml and 300 LD_{50} per mg N (average values).

(b) *Ultrasonic treatment of water suspension.* A 2.5% suspension of organisms in distilled water was subjected to ultrasonic treatment in a 9 KC oscillator for one hour at 0°C. The extract, after removal of the bacterial debris by centrifugation at 4000 r.p.m. for one hour at 0°C, contained 1080 LD_{50} per ml and 600 LD_{50} per mg N. The original water extract in this experiment contained 230 LD_{50} per ml and 250 LD_{50} per mg N[‡]. This represented approximately a 5-fold increase in toxin and only a 2-fold increase in nitrogen. Nevertheless, the ultrasonic method was not used for further extraction of the toxin because of the difficulties involved in processing large volumes of material.

(c) *Extraction of organisms with $CaCl_2$.* In a previous report(9), it was shown that the presence of 0.05 M to 0.1 M $CaCl_2$ in a 2.5% suspension of *H. pertussis* removed large amounts of mucin-like impurities which greatly hindered preparative procedures. The extracts contained all of the toxin originally present. In the present study, the extract obtained from a 2.5% suspension of organisms in 0.05 M $CaCl_2$ at pH 6.5, after removing the bacterial debris by centrifugation at 4000 r.p.m. for one hour at 0°C, contained

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[†] This strain was obtained from Lederle Laboratories Division, American Cyanamid Co.

12. Ross, O. A., unpublished data.

13. Robbins, K. C., and Pillemer, L., *J. Immunol.*, in press.

14. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

15. Gomori, G., *J. Lab. and Clin. Med.*, 1942, v27, 955.

16. Longsworth, L. G., *Ind. Eng. Chem.*, 1946, v18, 219.

[‡] This experiment was carried out with a batch of organisms low in toxin.

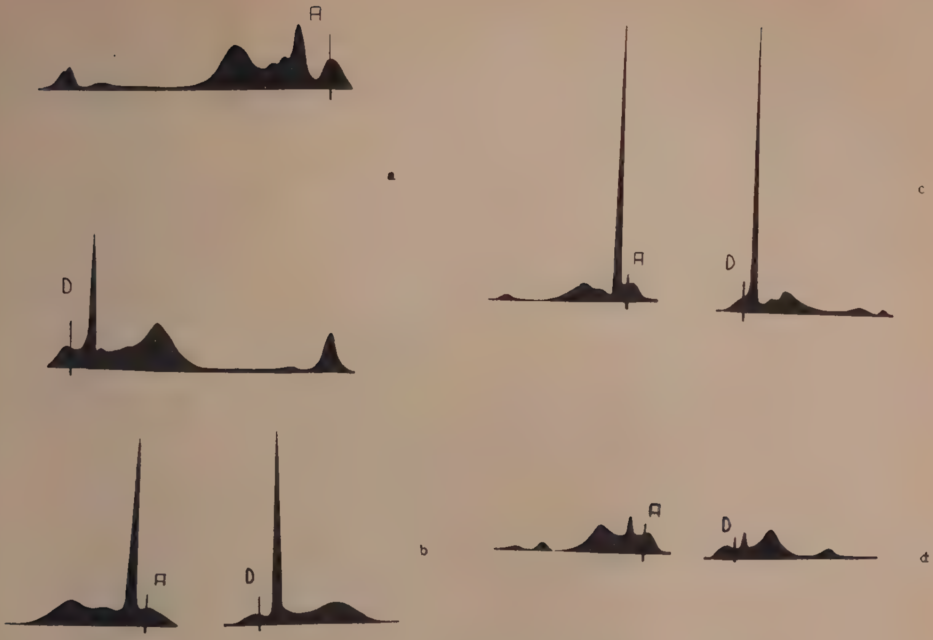

FIG. 1.
Electrophoretic patterns of extracts and fractions of *H. pertussis* in pH 8.6 veronal buffer, ionic strength of 0.10. (a) Water extract. (b) 0.05 M $CaCl_2$ extract. (c) Fraction PI. (d) Fraction PII.

280 LD_{50} per ml and 400 LD_{50} per mg N (average values). This represented an average yield of 78% of the water extract with a purification factor§ of 1.3. The N/P ratio was found to be 19.8. Similar results were obtained with 0.025 and 0.1 M $CaCl_2$ extracts. The optimum pH for extraction of the toxin in 0.05 M $CaCl_2$ was found to be 6.5. Higher hydrogen-ion concentrations gave lower yields. The 0.05 M $CaCl_2$ extract at pH 6.5 was used as the starting material for fractionation.

(d) *Separation of toxin from 0.05 M $CaCl_2$ extract(1). Fraction PI.* Orientation studies revealed that the toxin can be optimally precipitated from the 0.05 M $CaCl_2$ extract at pH 4.4 in 15% methanol, ionic strength of 0.13 at –5°C. The pH was adjusted with hydrochloric acid. The fractionation methods employed have been described in previous publications(13,17,18). The precipitate was dissolved to one-fifth of the original volume in 0.30 M sodium acetate at pH 7.0 and the solution clarified by centrifugation at 4000 r.p.m. at 0°C for one hour. This fraction (PI), which is 5x concentrated, contained 1140 LD_{50} per ml and 1630 LD_{50} per mg N (average values). This represents an average yield of 82% of the 0.05 M $CaCl_2$ extract with a purification factor of 5.4. The N/P ratio was found to be 55.5. In this step, small changes in methanol concentration influenced the solubility of the toxin and the impurities to a greater extent than did small changes in the ionic strength and pH.

(2) *Fraction PII.* Preliminary studies indicated that the toxin could be optimally

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§ Purification factor = $\dfrac{LD_{50} \text{ per mg N in fraction}}{LD_{50} \text{ per mg N in water extr.}}$

17. Pillemer, L., Wittler, R. G., Burrell, J. I., and Grossberg, D. B., *J. Exp. Med.*, 1948, v88, 205.

18. Pillemer, L., and Robbins, K. C., *Ann. Rev. Microbiol.*, 1949, v3, 265.

reprecipitated from PI in aqueous solution at pH 5.3, ionic strength of 0.15 at 0°C. The precipitate was removed by centrifugation and was dissolved to the original volume of PI in 0.15 M sodium acetate at pH 7.0 and the solution was clarified by centrifugation. This fraction (PII) contained 920 LD_{50} per ml and 5260 LD_{50} per mg N (average values). This represents an average yield of 81% of PI with a purification factor of 17.5. The overall yield at this step was 51%. The N/P ratio was found to be 43.1.

(e) *Electrophoretic analysis.* Electrophoretic patterns of the water extract, the 0.05 M $CaCl_2$ extract, Fraction PI and Fraction PII from *H. pertussis,* strain No. 29, are shown in Fig. 1. The most active fraction, PII, was found to be heterogeneous.

Discussion. It has been found that the most favorable conditions for the separation of pertussal toxin involve extraction of organisms dried from the frozen state with 0.05 M $CaCl_2$ at pH 6.5 followed by precipitation at pH 4.4 in 15% methanol, ionic strength of 0.13 at −5°C and reprecipitation at pH 5.3, ionic strength of 0.15 at 0°C. The most active fraction, PII, contained 5260 LD_{50} per mg N which represented a 17.5-fold purification over the water extract with an average overall yield of 51%. Fraction PII was found to be heterogeneous by electrophoretic analysis.

The relationship of toxin found in strain No. 29 to that found in true phase I strains has not been definitely established. Strain No. 29 has been shown to satisfy only one of the serological criteria for a phase I organism, namely agglutination by a phase I antiserum(13). This organism contains protective bacterial antigens against the phase I strain used in the mouse protection test(13). The bulk of the available experimental evidence suggests that the toxin found in the variant strain No. 29 is the same as that found in all strains of *H. pertussis.*

Summary. Methods have been described for the separation of pertussal toxin from a 0.05 M $CaCl_2$ extract of *H. pertussis,* strain No. 29, dried from the frozen state. The most active fraction contained 5260 LD_{50} per mg N and was found to be heterogeneous by electrophoretic analysis.

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Influence of Feeding Schedule on Nitrogen Utilization and Excretion.
(17815)

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The object of the experiment reported in this paper is to find the answer to a practical question of nutrition: With a given total dietary intake does the feeding schedule have any influence on the efficiency of N utilization, and if so, is it more efficient to have 2, 3 or 4 feedings a day?

According to the current concept of dynamic equilibrium of body constituents, tissue proteins are continually broken down into amino acids and new tissues are continually formed from amino acids. When the rate of protein formation equals the rate of protein breakdown, the body is in protein equilibrium. Besides the reversible reaction between proteins and amino acids, the latter are continually removed irreversibly from the metabolic pool by excretion as such and by conversion into nitrogenous waste products, chiefly urea. The rates of removal of amino acids from the metabolic pool, whether by formation of protein or urea, or by excretion, should vary with the concentration of the assorted amino acids, other things being equal. If the rate of removal of amino acids equals the rate of replenishment from the digestive tract, the concentration of amino acids in the metabolic pool would remain con-

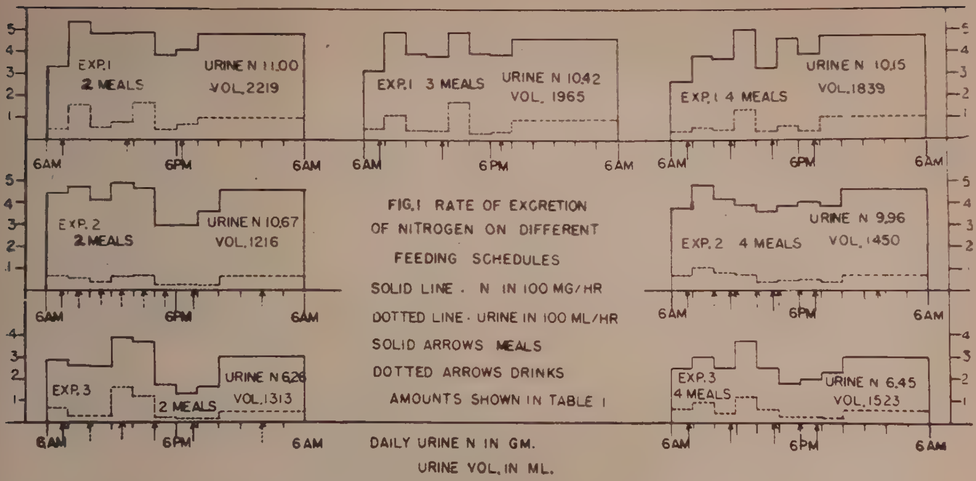

Fig. 1.

stant and the body would be in N balance. Actually, the concentration of amino acids in the metabolic pool must fluctuate with feeding. With this fluctuation, the rates of removal of amino acids by whatever process must fluctuate also. The well known specific dynamic effect of proteins is largely, if not wholly, an expression of the increase in the rate of metabolism of amino acids, as their concentration in the pool is raised by feeding. Following each meal, there is an increase in amino acid concentration in blood, which falls again to the pre-feeding level after several hours. In each day the amino acid concentration in the metabolic pool rises and falls like a wave. With each wave the rates of removal of amino acids rise and fall also.

While the increased catabolism of protein at the trough of the wave may be compensated by the increased anabolism at the crest, the increased loss of amino acids by excretion and by urea formation at the crest, is not compensated by a reverse reaction at the trough. It would seem from these considerations that the amount of amino N irreversibly removed from the pool would depend on the frequency and amplitude of the waves of amino acid concentration which in turn depends on the feeding schedule. Given the same total intake of protein, the loss of N by irreversible removal of amino acids should be smaller and consequently the efficiency of N utilization should be higher with frequent feedings of light meals than with fewer feedings of heavy meals, other things being equal. The experiment to be reported is an attempt to test this hypothesis. Incidentally, it throws some light on the question of the rhythm of N metabolism.

Experimental. The subject was a normal adult who had maintained his body weight (68.5 kg) for many months and was presumably in N equilibrium. The diet of free choice before the experiments contained 78 g protein and supplied 2500 calories. The diet in Exp. 1 was constructed to give the same amounts of protein and calories.

Exp. 1 consisted of periods of 4 days each. The diet was exactly the same throughout the experiment, but it was divided into 2, 3 and 4 meals, approximately equal with respect to protein and calories, in the first, second and third periods respectively.

Exp. 2 was performed 3 months after Exp. 1. The diet of free choice before this experiment also contained 78 g protein but the calorie value (2300) was a little lower. The diet in Exp. 2 was constructed accordingly.

Exp. 3 was performed 2 weeks after Exp. 2. In the interval between these 2 experiments, the protein intake was gradually reduced from 78 to 50 g, the calorie intake re-

TABLE I.
Composition of Diets.

	Exp. 1	Exp. 2	Exp. 3
Orange, E. P.	260	280	240
Lettuce, ”	140	140	140
Tomato, ”	200	—	—
Grape, ”	170	170	—
Apple, ”	—	170	200
Rice	100	68	40
Bread	72	120	96
Eggs, E. P.	108	108	108
Ham	200	130	50
Milk	250	400	230
Butter	30	50	40
Sugar	30	30	30
Jam	—	—	215
Grape juice	250	—	—
Mayonnaise	—	—	21
Total protein	78.4	78.3	49.5
” calories	2540	2250	2300
Water intake			
2-meal	2650*	2420†	2000‡
3- ”	2690	—	—
4- ”	2550	2320†	2250§

* Including 360 ml in drink at 1:30 p.m.

† Including 6 drinks of 100 ml each at times shown in Fig. 1.

‡ Including 4 drinks of 200 ml each at times shown in Fig. 1.

§ Including 2 drinks of 350 ml each at 11:30 a.m. and 3:30 p.m., 200 ml at 6 p.m., and 150 ml at 7:30.

maining the same. With 3 feedings a day, nitrogen balance was attained before Exp. 3 began. In this experiment, the diet contained 50 g proteins and supplied 2300 calories.

Exp. 2 and 3 each consisted of two 4-day periods. The diet was divided into 2 and 4 meals approximately equal with respect to proteins and calories, in the first and second periods respectively.

The composition of the diets is shown in Table I. In Exp. 1 and 2, 70%, and in Exp. 3, 65%, of the protein were of animal origin. The feeding schedules are shown in Fig. 1. All foods were weighed to the gram. The protein and calorie intakes were calculated from standard food tables, but the nitrogen content of the ham used in each experiment was determined by analysis. Throughout the experiments, the same mode of living was maintained. There was no change of body weight and there was no untoward event of any kind. In all the experiments, the first day of each period was considered as preliminary. For the following 3 days, urine was collected at 2-hour intervals during the day and as often as convenient during the night. In Exp. 2 and 3, the 3-day feces marked with charcoal were collected. Nitrogen was determined in the urine and feces in the usual manner. The results are shown in Table II.

Discussion. It will be noted that in Exp. 1 the N excretion diminished with increasing number of feedings. In Exp. 2 the N excretion in the 2-meal period was also higher than that in the 4-meal period. The differences are, however, small in both experiments. If the results of these experiments in which the N intakes were the same, are combined, the mean N excretions in the 2-meal and 4-meal periods were 10.83 ± 0.19 and 10.06 ± 0.18 g respectively. The difference between them was 0.77 ± 0.26 g, which was barely significant statistically. This difference may be taken as the difference in N utilization, if the efficiency of absorption is assumed to be the same with different feeding schedules.

There was a slight difference in fecal N between the 2-meal and 4-meal periods in Exp, 2. This difference is uncertain on account of the well known difficulty of separating the feces of different periods. If this difference is taken into account and assuming the same values for Exp. 1 as for Exp. 2, the difference in N balance in both experiments would be 0.54 g in favor of the 4-meal schedule.

If the loss of N through the skin is taken as 0.36 g per day(1), there would be a slight negative balance in the 2-meal period and a slight positive balance in the 4-meal period in both experiments. And with 3 meals there would presumably be exact balance.

In Exp. 3, there was no significant difference in N balance between the 2-meal and 4-meal periods.

It may be concluded, therefore, that with a liberal protein intake of 1.14 g per kg, there is a definite indication of a higher efficiency of N utilization with the 4-meal schedule as compared with the 2-meal schedule. With a

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1. Mitchell, H. H., *Arch. Biochem.*, 1949, v21, 335.

TABLE II.
Influence of Feeding Schedule on Nitrogen Balance.

	Exp. 1			Exp. 2		Exp. 3	
Level of protein intake, g	78			78		50	
” ” calorie ”	2500			2300		2300	
No. of feedings	2	3	4	2	4	2	4
Food N, g	12.53	12.60	12.54	12.50	12.65	7.92	7.92
Fecal N, g				1.80	2.12	1.68	1.55
N absorbed, g				10.70	10.53	6.24	6.37
Urine N, g	11.00	10.42	10.15	10.67	9.96	6.26	6.45
P. E.*	±.32	±.07	±.17	±.24	±.35	±.19	±.17
Diff. between 2 and 4 feedings	.85 ± .37			.71 ± .43		—.19 ± .25	
N balance, g				+.03	+.57	—.02	—.08
Diff. between 2 and 4 feedings				.54		.10	

$$* \text{P.E.} = 0.67 \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

lower protein intake (0.73 g per kg) no significant difference in N utilization was observed. This is understandable, as with lower protein intake, the height of the amino-acid concentration reached in the metabolic pool after feeding will be lower and consequently the irreversible loss by excretion and by urea formation will be smaller. The pattern of N excretion differed with different feeding schedules. In spite of some variation due to uncontrollable factors, each feeding schedule produced a more or less characteristic pattern, which repeated itself with remarkable uniformity from day to day in each period. There were one or more crests and troughs of N excretion during the day with one crest at night (not shown in the composite diagram in Fig. 1 which shows the average rate of excretion from 8 or 10 P.M. to 6 A.M.)

In Exp. 1 there seemed to be as many waves of N excretion as there were feedings, suggesting a rise of amino acid concentration after each meal. In Exp. 2 and 3, however, there seemed to be, broadly speaking, only one crest in the morning or early afternoon and a trough between 4 and 8 P.M. The difference in the N excretion pattern observed between Exp. 1 on the one hand and Exp. 2 and 3 on the other, even when the periods with the same number of feedings are compared, may be due to the difference in the level of N or water intake, but possibly also to other uncontrolled factors (such as salt intake and perspiration) which render theoretical interpretation of the patterns difficult if not impossible. In any case the variations of N excretion do not necessarily correspond to variations of amino acid concentration in the metabolic pool. It is well known that N excretion lags behind N metabolism. In Exp. 1 the crest of N excretion came too soon to be accounted for by increased N metabolism. Furthermore, it will be noted that the variations of N excretion were almost always accompanied by corresponding variations in urine volume. Since the rate of excretion of urea is known to increase with urine volume, it is impossible to tell whether an increase in N excretion is due to an increase in urea formation or to an increase in excretion of urea already formed.

There has been some discussion in the literature on the rhythm and diurnal cycle of N metabolism. In adults kept in bed and receiving 3 equal meals a day, Forsgren and Schnell(2) observed a rise of N excretion in the morning and forenoon with a maximum after midday and a fall in the afternoon and evening with a minimum after midnight. These authors ascribed this variation of N excretion to a rhythmic function of the liver which was supposed to have its assimilatory phase during the night and its dissimilatory

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2. Forsgren E., and Schnell, R., *Acta Med. Scand.*, 1934, v82, 155.

phase during the day, irrespective of meal hours and sleep. This theory was supported by Gerritzen(3) who reported some observations on healthy young adults, some of whom drank hourly and had meals at the usual hours while others drank and ate the same amount of food hourly.

From observations on children (5-10 years of age) receiving 4 meals at 6-hourly intervals, Manchester(4) found the maximum excretion of N to be between 6 A.M. and 12 noon and the minimum between 12 midnight and 6 A.M. Examination of the data reported by these authors showed that the variation in N excretion was always accompanied by a corresponding variation in urine volume. The so-called rhythm of N excretion may be simply a rhythm of water excretion which depends not only on food and water intake but on a host of other factors which impinge on the complex nervous and hormonal control of water metabolism. Unless the rate of water excretion is fairly uniform, it is impossible to locate the real crest and trough of N metabolism or indeed to decide whether there is a cycle or rhythm of N metabolism independent of feeding and other routine of living.

Although the present experiment was originally conceived as a test of the influence of feeding schedule on N utilization through its effect on the amino acid concentration in the metabolic pool, it is realized that N balance experiments can reveal not this effect alone but rather the net effect on a whole series of processes from digestion to excretion. Furthermore, as the present experiment has been performed on a single subject and the number of observations is relatively small, the conclusions must be regarded as tentative. However, the result does indicate that feeding schedule is a factor to be considered in metabolism experiments.

Summary. A normal adult in N equilibrium was given the same diet divided into 2, 3, and 4 approximately equal meals in different periods. With a liberal protein intake (1.14 g per kg), there was a definite indication of a higher efficiency of N utilization with 4-meal than with 2-meal schedule, but the difference was not appreciable to be of practical importance. With a lower protein intake (0.73 g per kg), no significant difference in N utilization was observed.

With the same total intake of food, the pattern of N excretion differed with different feeding schedules and with total N and water intakes. This finding casts doubt on the existence of a diurnal cycle or rhythm of N metabolism irrespective of water intake and feeding schedule.

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3. Gerritzen, F., *Acta Med. Scand.*, 1936, v89, 101.

4. Manchester, R. C., *J. Clin. Invest.*, 1933, v12, 995.

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Effect of Adrenocorticotropic Hormone and Cortisone Therapy on Human Plasma Aminopeptidase Activity.* (17816)

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Recent reports have indicated that a considerable increase in serum peptidase activity occurred following the injection of ACTH or adrenal cortical extract in mice(1) and that much less striking increases in various serum peptidases appeared after the administration

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TABLE I.
Plasma LGGase Activity of Normal Subjects.†

Subject	Age	Sex	Date	K°_{LGG}*	
				60 min.	120 min.
T.	32	M	1/3/50	.067	.067
			5	.073	.070
			6	.067	.067
			11	.073	.067
J.	23	M	2/17	.053	.054
			3/16	.055	.052
C.	23	F	16	.061	.055
M.	33	M	16	.041	.039
L.	26	M	16	.059	.063

* Enzyme-substrate mixtures consisting of 0.5 ml of 0.1 M LGG, 0.5 ml plasma, 0.5 ml of 0.02 veronal buffer (pH 7.8), and 0.5 ml 0.85% NaCl were maintained at 37.8°. K°_{LGG} (see text) was calculated from the per cent hydrolysis of LGG of samples withdrawn from enzyme-substrate mixtures at 60 and 120 minutes after the start of hydrolysis.

† This group will be expanded considerably when greater quantities of LGG become available.

of ACTH to normal men(2). We have been unable to confirm these observations in the rat and mouse(3), and have extended these investigations to include patients treated with ACTH and Cortisone.‡

Methods. Ten ml of venous blood, from patients usually fasted, were mixed with 0.1 ml of heparin solution,§ centrifuged and the clear *non-hemolyzed* plasma removed. Enzymic hydrolysis of L-leucylglycylglycine (LGG) by human plasma (LGGase activity) was followed essentially as described elsewhere(3). Per cent hydrolysis was determined using a ninhydrin photometric procedure(4). Plasma LGGase activity was represented by the constant, K°_{LGG}, which is defined as the per cent hydrolysis of LGG per minute for 0.1 ml plasma per ml of hydrolysis mixture.

Results. In a small series of controls it was found that plasma K°_{LGG} for normal subjects, fasted or fed, varied from approximately 0.04 to 0.08, as indicated in Table I.

In Figs. 1 and 2 are shown changes in

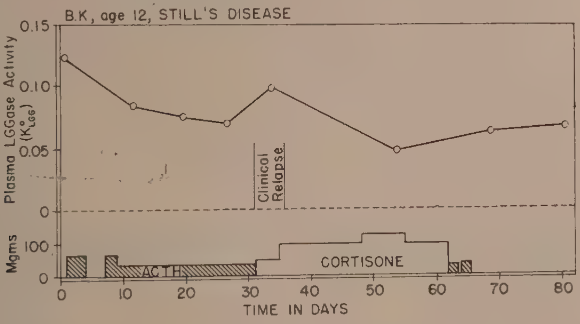

FIG. 1.
Changes in plasma LGGase activity occurring with ACTH and Cortisone therapy in a patient with Still's disease.

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1. Holman, H. R., White, A., and Fruton, J. S., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 196.

2. Sayers, G., Burns, T. W., Tyler, F. H., Jager, B. V., Schwartz, T. B., Smith, E. L., Samuels, L. T., and Davenport, H. W., *J. Clin. Endocrin.*, 1949, v9, 593.

‡ More complete metabolic studies on these patients await future publication.

§ The small dilution error thus introduced has been ignored.

3. Schwartz, T. B., and Engel, F. L., *J. Biol. Chem.*, 1949, v180, 1047.

4. Schwartz, T. B., and Engel, F. L., *J. Biol. Chem.*, 1950, v184, 197.

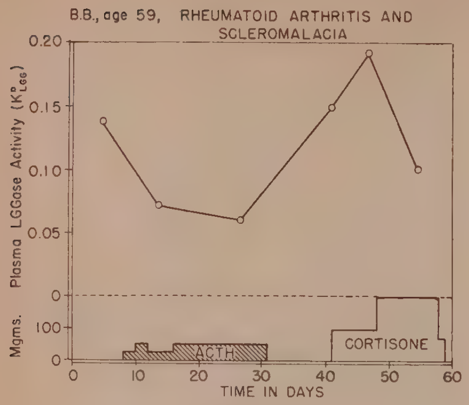

FIG. 2.

Changes in plasma LGGase activity occurring with ACTH and Cortisone therapy in a patient with rheumatoid arthritis and scleromalacia.

plasma K^0_{LGG} which occurred during treatment with ACTH and Cortisone. Note that pre-treatment levels are high and fall with adequate hormonal therapy. These results are, in part, similar to those of Stern and associates(5) who have shown in patients with fractures that cobalt-activated serum LGGase activity falls from high to normal levels with healing.

The data shown in Table II reflect other observations of interest. In patients Y., F., H., and N. no clinical improvement was manifested during adequate therapy with potent hormonal preparations and plasma LGGase activities did not change significantly from pre-treatment values.

That intense chronic adrenal hypersecretion may not increase plasma LGGase activity is illustrated by patient C. with Cushing's syndrome, whose plasma K^o_{LGG} was repeatedly normal. Similarly, in patient A. with chronic adrenal cortical insufficiency plasma K^o_{LGG} was within the normal range. Finally, in another patient (L.) with Addison's disease complicated by a migrating polyarthritis, plasma LGGase activity was significantly elevated. It should be mentioned that LGGase activity of human erythrocytes have been studied, normal values ranging from 4.8 to 6.2. No consistent or profound

TABLE II. Observations on Human Plasma LGGase Activity.

Subject	Age	Sex	Diagnosis	Hormonal therapy	Duration of therapy, days	K^o_{LGG}* 60 min.	120 min.	Clinical status
Y.	8	F	Acute leukemia	0	—	.056	.058	Essentially unchanged.
				ACTH	2	.049	.051	
				,,	6	.052	.050	
				,,	11	.033	.034	
				,,	14	.057	.055	
F.	50	M	Intestinal	0	—	.132	.132	,, ,,
			lipodystrophy	Cortisone	14	.122	.121	
H.	49	M	Malignant hyper-	0	—	.30	.30	Died 5 days after start of therapy.
			tension with uremia	ACTH	3	.36	.31	
N.	6	M	Acute leukemia	0	—	.28	.26	Died 8 days after start of therapy.
			without uremia	Cortisone	2	.32	.30	
				,,	6	.30	.30	
C.	28	F	Cushing's syndrome	0	—	.075	.075	Unchanged (4 day interval between samples)
				0	—	.077	.074	
A.	45	F	Addison's disease	Desoxycorticosterone	Continuous	.072	.067	
L.	42	M	,, ,,	,,	,,	.103	.100	Acute migrating arthritis.

* See footnote, Table I.

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5. Stern, K., Cullen, A. M., and Barber, V. T., *J. Clin. Invest.*, 1949, v28, 419.

changes in erythrocyte K^{o}_{LGG} occurred in this group of patients during ACTH or Cortisone therapy.

Summary and conclusions. 1. In a small control series the normal range of human plasma L-leucylglycylglycine-splitting (LGGase) activity has been established.

2. In 2 patients who responded to ACTH and Cortisone therapy, the pre-treatment LGGase activities were abnormally *high* and, concurrent with clinical remissions, plasma LGGase activities fell to normal levels only to rise again with onset of clinical relapses after cessation of therapy.

3. In 4 patients who did not respond to hormonal therapy, no change in plasma LGGase activity occurred irrespective of whether pre-treatment levels were high or normal.

4. Plasma LGGase activity was normal in a patient with Cushing's syndrome and in a patient with Addison's disease but was significantly elevated in a second patient with Addison's disease complicated by arthritis.

5. These studies lend credence to the hypothesis that changes in plasma LGGase activity reflect changes in clinical status and are not necessarily mediated through the adrenal cortex.

We wish to thank Mrs. Catherine Towbin for technical assistance provided during the course of this investigation.

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Application of the Feulgen Reaction to the Laboratory Diagnosis of Smallpox.* (17817)

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The elementary bodies of smallpox were first described by Buist, and then independently by Paschen(1). Methods for microscopical diagnosis of these bodies have been proposed by several investigators. Van Rooyen and Illingworth(2) were able to differentiate variola from varicella by staining the elementary bodies with basic fuchsin as proposed by Paschen. Herzberg(3) recommended victoria-blue for the same purpose. Recently Nagler and Rake(4) reported on the use of electron microscopy for the diagnosis of smallpox. Phase contrast microscopy has also been suggested for this purpose (Barer)(5). None of these methods provides means of distinguishing elementary virus bodies from other small particles. Bland and Robinow(6) have shown that the elementary bodies of vaccinia, and the corresponding inclusion bodies react positively to Feulgen's method of staining desoxyribonucleoproteins. In view of the great similarity, or possible identity, of the viruses of vaccinia and variola, we applied the Feulgen reaction to the study of the variola virus. The present report deals with the use of the Feulgen reaction as a quick diagnostic test for smallpox.

Technic. Skin lesions, preferably fresh vesicles or papules are opened with a sharp instrument, laying free the base of the lesion.

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* The author is grateful to Dr. Klasmer of the Jerusalem Office of the Israel Health Department, for his continuous collaboration and help in obtaining most of the material used in this study.

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2. VanRooyen, C. E., and Illingworth, R. S., *Brit. Med. J.*, 1944, v2, 526.

3. Herzberg, K., *Klin. Wchnschr.*, 1936, v15, 1385.

4. Nagler, F. P. O., and Rake, G., *J. Bact.*, 1948, v55, 45.

5. Barer, R., *Nature*, 1948, v162, 251.

6. Bland, J. O. W., and Robinow, C. F., *J. Path. and Bact.*, 1939, v48, 381.

Scrapings of the base are then rubbed on the surface of a clean and smooth glass slide. The slide is fixed in ether-alcohol (1:1) mixture for a few minutes (in a Coplin jar), and then left to dry. The smears are stained according to Feulgen's method as described by Lillie(7), and counterstained with fast green FCF. They may be either dried in the air after having been washed in water, or else dehydrated in alcohols, cleared in xylene and mounted, if permanent preparations are required. Fast green FCF was found very useful in providing contrast to the light purple or red staining produced by the Schiff reagent. The study was performed with an ordinary laboratory microscope with a magnification of x 1000 diameters.

Results and discussion. In the scrapings of smallpox cases multiple Feulgen positive granules were found free, as well as within epithelial cells. Not all the cells contained the granules. Those which did, contained them in great numbers and density. The granules were sometimes found lying free in great numbers in the neighborhood of burst epithelial cells. The average size of the granules as determined by microphotography, was found to be about 0.3m. (Fig. 1). In the scrapings of varicella cases no such granules were found. Some cells contained a few minute granules, which could hardly be seen. The granules appeared smaller than those of smallpox, and were never found in great numbers or lying close to each other. As was noted by van Rooyen and Illingworth the elementary bodies of varicella are relatively scanty and hard to detect.

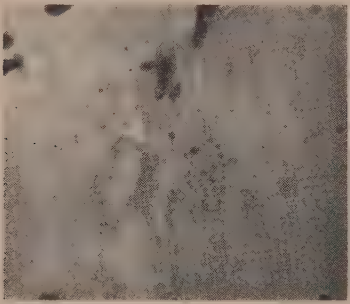

FIG. 1.
Elementary bodies of smallpox in a smear, stained with Feulgen's method. × 1200.

The microscopical appearance of the elementary bodies described above tallies with the description given by previous authors in all but one respect:

Our study of variola scrapings suggests that the elementary bodies are surrounded by a thin clear halo. The halo is reminiscent of that found around Guarnieri bodies in the cytoplasm of cells. It is not clear whether the halo is real, or a diffraction effect. Its possible relationship to the halo found around the Guarnieri bodies warrants further investigation.

The method has been tested so far in the following: 1. Two clinically certain cases of smallpox, whose diagnosis has been corroborated by various laboratory methods. In both these cases a positive result was obtained with the Feulgen procedure. 2. Nine cases in which there had been close contact with proved smallpox cases, and in which a mild disease, clinically similar to, but not identical with chickenpox had been observed. Eight of these gave a positive test for smallpox with the Feulgen procedure. The diagnosis of smallpox has been later confirmed by other laboratory methods (chorio-allantois cultures, and in some cases,—Paul's test). 3. Twenty-one clinically certain cases of chickenpox. Nineteen of these could be diagnosed with the Feulgen procedure as "smallpox negative". Two were doubtful. In 14 of the cases typical elementary bodies of chickenpox were found.

Comment. The Feulgen method affords means of visualising smallpox elementary bodies. The method has a distinct advantage over other methods of staining: It allows a clear differentiation of virus particles from dirt and extraneous material. Only virus particles and nuclear chromatin of cells are stained, while dirt, and cytoplasmic matter, which do not contain desoxyribonucleic acid give no positive reaction. The method allows a quick laboratory diagnosis of smallpox and does not require any complicated apparatus. The main points in the differential diagnosis of smallpox from chick-

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7. Lillie, R. D., *Histopathologic Technic*, Blakiston, Philadelphia, 1948.

enpox remain those defined by previous observers: (1) Size of the elementary bodies, (2) their number inside cells and lying free, (3) their distribution. The method was found useful in the differential diagnosis of variola from varicella. Its application for establishing a positive diagnosis of chickenpox presents certain drawbacks: (a) Elementary bodies are not found in general in chickenpox vesicles 4-7 days old, and sometimes even in more recent lesions. (b) Varicella elementary bodies seem to lie near the limit of visibility, and their detection requires an effort on the part of the microscopist.

It should also be noted that the Guarnieri bodies in human skin and other tissues are Feulgen positive. Furthermore the Feulgen technic seems to be a method of choice for the study of Guarnieri bodies in tissue sections(8). The method was also applied with good results to smears and sections of chorio-allantois cultures of smallpox virus. Rickettsiae could also be stained and differentiated from extraneous matter by the same technic.

Summary. A method for staining elementary bodies of smallpox is described. The method consists of the application of Feulgen's method for desoxyribonucleic acid to smears made of scrapings of the bottom of skin lesions. The procedure seems to afford the means for a quick diagnosis of smallpox, and allows differentiation of virus particles from extraneous material.

Thanks are also due to Dr. Sagher of the Skin department of this hospital, to Dr. Nelken of the Worker's Sick Fund Clinic, Jerusalem and Miss Rabinowitch, of the Bacteriology Department, Hebrew University, for additional material.

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8. Wolman, M., not yet published.

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Reversal of Aminopterin Inhibition in the Chick Embyro with Desoxyribosides.* (17818)

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Aminopterin (4 aminofolic acid, 4-aminopteroylglutamic acid)(1) is a highly toxic substance for certain microorganisms(1,2), mice (3), guinea pigs(4), chicks(5), rats(5,6), and man(7). In certain microorganisms (*e.g.* *Streptococcus faecalis*)(1) and to a lesser extent in certain animals (*e.g.* mice)(3), its inhibitory effects are prevented by relatively high amounts of folic acid; for this reason, and because of its close structural similarity to the vitamin, aminopterin is considered almost universally to act *in vivo* as an antagonist to folic acid. Especially in animals, however, and at relatively high levels of administration in all organisms, it has not proved possible to counteract the toxic effects of aminopterin with folic acid(3,7), *i.e.* the antagonistic relationship is not fully competitive. If, under these circumstances, the inhibited reaction could be made non-essential by furnishing its product, alleviation of the inhibition might be expected(8,9). Nothing is known of the processes which folic acid antagonists inhibit in animal tissues. In lactic acid bacteria, however, folic acid is thought to be essential for synthesis of thymine and purine bases, since a mixture of these substances replaces folic acid in the nutrition of several organisms(10,11), and counteracts growth inhibition of *Lactobacillus casei* by methylfolic acid(9).

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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2. Sauberlich, H. E., *Arch. Biochem.*, 1949, v24, 224.

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The present studies were undertaken to determine the effect of aminopterin on the developing chick embryo, and by testing the effectiveness of various compounds in reversing any observed effects, to throw some light on the mechanism of action of this inhibitor in animal tissues. Since these studies were initiated or, Karnofsky, *et al.*(12) have reported that aminopterin inhibits development of the chick embryo. High levels of folic acid (20 mg per egg) did not counteract these inhibitory effects.

Experimental. The procedures followed in injecting solutions and incubating the eggs were identical to those described by Cravens and Snell(13) in similar studies with desoxypyridoxine. All injections were in a total volume of 0.1 ml. At higher concentrations, where insolubility of aminopterin and folic acid required it, these compounds were dissolved and injected as their sodium salts.

Results. Data of Table I show the effect of injection of aminopterin prior to incubation on embryonic development. In summarizing these data, no attempt has been made to distinguish infertile eggs from those in which the embryo died prior to the appearance of blood. Definite blood formation was considered evidence that the embryo was at least in the second day of development at death. Control eggs showed a mortality of 11.7% at 0-1 day of development and the bulk of these embryos were infertile as judged by the appearance of the blastodisc (14). One hundred micrograms or more of aminopterin inhibited embryonic development completely. By gross examination, such eggs could not be differentiated from infertile eggs. As the amount of inhibitor was reduced, the age at death increased for a small percentage of the embryos. Ten micrograms per egg still produced death of all embryos at some stage of development before hatching; the highest amount injected which allowed any live chicks to hatch was 5 μg per egg. Even 2 μg per egg significantly increased mortality. These results demonstrate the extreme activity of aminopterin in inhibiting embryonic development. They agree well with those of Karnofsky *et al.*(12), who found the LD_{50} of aminopterin to be 3 μg per egg when injected on the fourth day of incubation. For subsequent work, 20 μg of aminopterin was chosen as a convenient amount which consistently produced 100% mortality, but allowed development to proceed sufficiently to permit detection of any favorable effects exerted by other factors. The effect of time of injection upon toxicity of this amount of the inhibitor was determined using 10 eggs per group. At three days of incubation the inhibitory effect is almost immediate, and 100% mortality still results, while at 6 days a delayed toxicity was evident which frequently produced death 2 to 6 days after the injection. Fifty per cent of these embryos eventually hatched. When injected at 9 or more days of incubation, this amount of the inhibitor is non-toxic.

The effects of several compounds injected in an attempt to counteract the inhibitory action of aminopterin are shown in Table II. All injections were made just prior to incubation. The compounds tested included folic acid, vitamin B_{12}, thymidine, hypoxanthine desoxyriboside, thymine, and hypoxanthine. An enzymatic digest of desoxyribonucleic acid,† and concentrates of the *Leuconostoc*

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9. Rogers, L. L., and Shive, W., *J. Biol. Chem.*, 1948, v172, 751.

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12. Karnofsky, D. A., Patterson, Priscilla A., and Ridgeway, Lois P., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 765.

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14. Kosin, I. L., *Poultry Sci.*, 1944, v23, 266.

TABLE I.
Effect of Aminopterin on Hatchability of Eggs.

Substance injected	Amt inj. μg	No. of eggs	% embryos dying at			
			0-1 day	2-5 days	6-20 days	% hatch
Aminopterin	1000	31	100			
”	500	15	100			
”	200	15	100			
”	100	15	100			
”	50	30	96.7	3.3		
”	20	115	73.9	22.6	3.5	
”	10	15	53.4	33.3	13.3	
”	5	30	36.7	23.3	16.7	23.3
”	2	15	26.7	13.3	6.7	53.3
Water (.1 cc)		129	11.6	6.2	11.6	70.5
None		128	11.7	4.7	2.3	81.3

citrovorum factor[‡] were also tested. None of these substances showed any significant effects on hatchability or mortality at the levels tested when injected without aminopterin.

Folic acid, vitamin B_{12}, and concentrates of the *Leuconostoc citrovorum* factor (LCF) were ineffective in overcoming effects of the inhibitor, both when injected singly and together. Thymdine in amounts of one or 2 mg per egg partially counteracted the inhibitory effects of aminopterin. Not only were some live chicks obtained, but consistent and pronounced shift toward longer survival of those embryos which died was evident. Thymine at equivalent levels and at higher levels (not recorded) was completely without ecect. Although hypoxanthine desoxyriboside alone was ineffective in overcoming aminopterin inhibition, it exerted a markedly favorable effect when injected together with thymidine. Hypoxanthine plue thymidine was no more effective than thymidine alone; thymine plus hypoxanthine desoxyriboside was ineffective. This pronounced action of the desoxyribosides in counteracting the effects of aminopterin is underscored by the high hatch obtained when an enzymatic digest of purified desoxyribonucleic acid, prepared in such a manner as to contain the desoxyribosides, was injected. It is of interest that combinations of thymidine with either folic acid or LCF were not significantly more active than thymidine alone, however, when all 3 substances were injected together, an apparent improvement in hatchability resulted.

Discussion. Inhibition of growth by any antivitamin results from failure of one or more catalytic reactions for which the corresponding vitamin (or a coenzyme derived from it) is essential. It has been generally assumed in such cases(8,16), that a metabolically inactive complex is formed between antivitamin and a specific protein which prevents formation of a metabolically active complex between the vitamin and this protein. Where such an inhibitor-protein complex is readily dissociable, inhibition can be alleviated by sufficient amounts of the appropriate vitamin. Where it is relatively undissociated, no reason to expect a competitive type of reversal exists(16). In the latter case, growth should still be possible if the *products* of the blocked reaction are supplied in a utilizable form(8,9).

The results of the above experiments are most readily interpreted in terms of this hypothesis. Since the pronounced inhibitory effects of aminopterin upon growth of the chick embryo cannot be reversed by folic acid, it may be assumed that complexes formed between aminopterin and the folic acid receptors

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[†] This digest was the $MgSO_4$ concentrate of enzymatically hydrolyzed desoxyribonucleic acid prepared by the method of Klein(15). It contains the mixed desoxyribonucleosides derived from the nucleic acid.

15. Klein, W., *Z. physiol. Chem.*, 1938, v255, 82.

[‡] We are indebted to Dr. C. A. Baumann and to Dr. T. R. Wood for concentrates of this factor.

16. Ackermann, W. W., and Potter, V. R., Proc. Soc. Exp. Biol. and Med., 1949, v72, 1.

TABLE II.
Effect of Various Compounds on the Inhibitory Action of Aminopterin.

Compound injected, amt.*	No. eggs	% embryos dying		% hatch
		0-5 days	6-20 days	
None	128	16.4	2.3	81.3
Water (.1 cc)	129	17.9	11.6	70.5
Aminopterin, 20 μg	115	96.5	3.5	0
” ”				
+ Folic acid, 1 mg	15	100	0	0
+ Thymidine, 1 ”	49	77.6	12.2	10.2
+ ” 2 ”	9	77.8	11.1	11.1
+ Thymine, .52 ”	10	100	0	0
+ Hypoxanthine desoxyriboside, 1 mg	10	100	0	0
+ LCF,† 33.3 μg	10	100	0	0
+ Vit. B_{12}, 1 μg	10	100	0	0
+ Thymidine, 1 mg + folic acid, .1 mg	20	85	10	5
+ ” 1 ” + ” ” .5 ”	20	60	20	20
+ ” 1 ” and Hypoxanthine desoxyriboside, 2 mg	19	36.8	31.6	31.6
+ Thymidine, 1 mg and Hypoxanthine, 1.1 mg	10	90	10	0
+ Thymine, .52 mg and Hypoxanthine desoxyriboside, 2 mg	10	100	0	0
+ Thymidine, 1 mg + LCF† 66.6 μg	10	80	10	10
+ ” 1 ” + ” 66.6 ” + Folic acid, .5 mg	10	50	10	40
+ ” ” .5 ” + LCF† 6.7 μg + Vit. B_{12}, 1 μg	10	100	0	0
+ Desoxyribonucleic acid digest, 5 mg	10	50	0	50

* Thymidine (m.p. 185-186°, $[\alpha]^{25^\circ}_{D}$ (1% solution in NaOH) = 31.2°) and hypoxanthine desoxyriboside, $[\alpha]^{25^\circ}_{D}$ (1% solution in water) = −20°) were isolated from sodium desoxyribonucleate. Other pure compounds were obtained from commercial sources.

† 66.6 μg of this concentrate was equivalent to approximately 1 ml of reticulogen (Lilly) in growth-promoting power for *Leuconostoc citrovorum*. Concentrates of this factor counteract the inhibitory effect of aminopterin for *L. citrovorum*(2).

are much less dissociable than those formed with folic acid itself. Since aminopterin inhibition is partially counteracted by thymidine, and more effectively counteracted by thymidine *plus* hypoxanthine desoxyriboside, these compounds would appear to be products, the formation of which is normally catalyzed by folic acid or its derivatives, and is prevented in the embryo by aminopterin at the levels employed here. The failure of hypoxanthine desoxyriboside to show any effect in the absence of thymidine is readily explicable if it is assumed that the first product to become limiting because of the aminopterin-induced folic acid deficiency is thymidine, the second, hypoxanthine desoxyriboside (or its nutritive equivalent), etc. The failure of mixtures of these two products completely to counteract aminopterin inhibition may result from partial failure of other metabolic reactions for which folic acid is required. This explanation of the observed facts implies that at concentrations of aminopterin considerably lower than 20 μg per egg, thymidine should be relatively more effective in counteracting aminopterin inhibition, while at considerably higher concentrations of aminopterin, mixtures of thymidine and hypoxanthine desoxyriboside might be relatively much less effective in counteracting inhibition than was found under the conditions used here. These implications have not been checked experimentally.

Previous work with bacteria had implicated folic acid in the synthesis of products related to thymidine and hypoxanthine desoxyriboside. In otherwise complete media, for example, the folic acid requirement of *Streptococcus faecalis* and of *Lactobacillus casei* is eliminated by addition of thymine (or thymidine) and any one of several purine bases to the medium(11); these same products counteract the inhibitory effects of methylfolic acid for *L. casei*(9). The present work demonstrates the existence of closely similar mechanisms in higher animals. Differences in detail exist, however. Thus, while thymine and hypoxanthine are effective in the cited bacterial systems, they are ineffective in the animal system, where the corresponding desoxyribosides show activity. A somewhat closer parallel to the results obtained in the chick embryo is provided by a strain of *Escherichia coli*, in which the inhibition of growth produced by aminopterin was counteracted by thymidine, but not by vitamin B_{12}, thymine, hypoxanthine or hypoxanthine desoxyriboside(17). Similarly, with *Leuconostoc citrovorum*, thymidine counteracts aminopterin inhibition(2) and purine bases are required in the medium to obtain this response(18). With this organism, however, concentrates of the *L. citrovorum* factor are highly active in counteracting aminopterin inhibition(2). Current evidence indicates that this factor is a metabolically active form of folic acid(2,19); however, at the levels available for testing, it was ineffective in counteracting aminopterin inhibition of embryonic growth under our conditions. Larger amounts might prove effective.

The decreased toxicity observed for aminopterin with increasing age of the embryo is similar to that observed previously for desoxypyridoxine(13), and may have a similar explanation. It was pointed out earlier that the specific aminopterin-protein complex presumably formed in the embryonic tissue must be relatively undissociable. Since the amount of this specific protein (enzyme) present in the embryo increases with embryonic development, an increase in the amount of aminopterin required for inhibition, *i.e.* an apparent decrease in inhibitory activity, might be expected. Ackermann and Potter(17) have discussed the occurrence of inhibitors of this nature, where the magnitude of the inhibitory effect is dependent upon the amount of enzyme present in the system.

Summary. Aminopterin, injected prior to, or early in, the incubation period, is highly toxic to the chick embryo. Ten micrograms or more of this compound produce 100% mortality; 100 μg or more per egg completely inhibit embryonic development. The inhibitor becomes relatively less toxic as embryonic development progresses; at nine days or more, twenty micrograms is non-toxic. The same amount injected at three days results in almost immediate embryonic death.

Thymidine partially counteracted the inhibitory effects of aminopterin. A combination of hypoxanthine desoxyriboside and thymidine was more effective than thymidine alone. In the absence of thymidine, hypoxanthine desoxyriboside was without effect. An enzymatic digest of desoxyribonucleic acid also counteracted aminopterin inhibition partially. Thymine, hypoxanthine, folic acid, vitamin B_{12}, and concentrates of the *Leuconostoc citrovorum* factor, alone and in various combinations, were ineffective in counteracting aminopterin inhibition.

These experiments indicate that aminopterin acts in animal tissues in part by preventing synthesis of thymidine and one or more of the purine desoxyribosides, and that these compounds are immediately essential for embryonic growth. Since aminopterin is thought to act by creating a deficiency in folic acid, the necessity for folic acid in synthesis of these compounds in animal tissues is implied.

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Rapid Production of Atherosclerosis by Administration of Uranium in Presence of Hypercholesterolemia. (17819)

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The recent demonstration by Holman that arterial disease may develop rapidly in man (1) and that in animals renal damage under certain conditions may result in the abrupt occurrence of arterial lesions(2) emphasizes the importance of determining those factors which are necessary for the rapid production of arterial vascular disease. This study is concerned with the rapid development of atherosclerosis in animals with hypercholesterolemia following the production of renal damage by the administration of uranium.

Methods. Male, albino rabbits between 10 and 12 months of age from a single colony were used. Cholesterol was administered by dissolving 5 g of cholesterol in ether and mixing this with Purina rabbit chow. The ether was evaporated and rabbits paired in cages were fed cholesterol by this technic 3 times a week. No supplementary feedings of vegetable greens were provided during this experiment. Water was freely available. Total cholesterol determinations using the Liebermann-Burchard reaction were made on ear vein blood each week. Kidney damage was produced by the subcutaneous administration of uranium acetate in a 10 mg per kg dose. Non-protein nitrogen determinations were made before uranium administration and just prior to sacrifice in animals receiving this chemical. All animals were killed by nembutal administration at the conclusion of the experiment and the thoracic and abdominal aortas were removed and fixed in formalin. Staining of the entire aorta with Sudan IV and confirmation of the presence of atherosclerosis by microscopic examination was carried out. The degree of atherosclerosis in the aorta was arbitrarily graded in the Sudan stained gross preparations on a 1 plus to 4 plus scale. Gross and microscopic examinations of the liver and kidneys were made in all experimental animals.

Four groups of animals were studied: (a) 10 animals with each pair fed 5 g of cholesterol 3 times weekly. These animals were sacrificed at the end of 3 weeks; (b) 10 animals fed Purina rabbit chow without additional cholesterol to which uranium acetate 10 mg per kg was given 3 to 14 days before sacrifice; (c) 14 animals maintained on the cholesterol feeding regime for 2 weeks, then given uranium acetate in the same dose and sacrificed from 2 to 14 days later; (d) 10 control animals from the same colony fed Purina rabbit chow alone.

Results. The data obtained are summarized in Table I. It will be noted that none of the control animals fed Purina chow alone and only 2 of the 10 normally fed animals receiving uranium acetate demonstrated significant atherosclerosis. Of the cholesterol fed group while 3 rabbits had atherosclerotic lesions of the aorta, these were of minimal degree. In the animals with hypercholesterolemia and uranium acetate administration 2 failed to demonstrate atherosclerosis but in the majority of rabbits in this group atherosclerosis was marked. All the cholesterol fed animals had some degree of fatty infiltration of the liver whereas the non-cholesterol fed animals did not. The kidneys of all rabbits receiving uranium had damage especially of the proximal convoluted tubules and to a much lesser extent of the glomeruli.

Discussion. The data herein presented does not permit identification of the factors responsible for the accelerated cholesterol deposition in the aorta in the presence of uranium acetate poisoning. However, the demonstration of the abrupt development of atherosclerosis in animals with hypercholesterolemia after the production of renal dam-

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1. Holman, R., *Fed. Proc.*, 1949, v7, 273.
2. Holman, R., *Am. J. Path.*, 1941, v17, 359.

TABLE I.
Summary of Data.

Diet	No. of animals	Avg initial wt, g	Initial avg total cholesterol mg/100 ml	Final avg total cholesterol mg/100 ml*	Avg N.P.N. mg/100 ml*	No. animals with aortic atheroma
Normal	10	2543	55 (29-80)§	—	20	0
Normal + uranium acetate 10 mg/kg†	10	2760	56 (35-84)	—	48	2 +1, +4
Cholesterol 3 wks	10	2968	59 (26-100)	1321 (647-1995)	—	3 all +1
Cholesterol 2 wks then uranium acetate 10 mg/kg‡	14	2647	44 (19-67)	499 (197-884)	118	12 Avg +2.6

* Determined just prior to sacrifice.
† From 3 to 14 days elapsed between uranium dose to sacrifice, avg 6.1 days.
‡ From 2 to 14 days elapsed between uranium dose to sacrifice, avg 5.9 days.
§ Numbers within parentheses indicate range.

age by this agent, further substantiates Holman's observation that "Arterial disease may be a matter of days not decades."(1)

Summary. Rabbits with dietary hypercholesterolemia of short duration develop aortic atherosclerosis abruptly after the production of renal damage by the administration of uranium acetate.

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Joint Action of Penicillin with Chloramphenicol on an Experimental Streptococcal Infection of Mice.* (17820)

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In the course of studies on the effects of antibiotics on enterococci it was observed that chloramphenicol interfered with the early bactericidal action of penicillin(1). In the presence of both penicillin and chloramphenicol the rate of bactericidal action was considerably slower than with penicillin alone. It was of interest to determine whether this apparent antagonism between two antibiotic agents is limited to an *in vitro* phenomenon or whether it could be demonstrated *in vivo*. Since enterococci are essentially nonpathogenic for small laboratory animals, a highly fatal infection with beta hemolytic streptococci was used in the present experiments.

Materials and methods. White Swiss mice weighing about 20 g, from a heterozygous stock, were used in all experiments. The infections were produced with a beta hemolytic, group A, type 3, streptococcus, strain C 203, kindly supplied by Dr. H. Eagle. The virulence of this organism was kept at high level by passage through mice once or twice weekly. Cultures were grown in 5% sheep blood broth at 37°C for 18 hours. The LD_{50} for intraperitoneal injection of mice consisted of 60-100 organisms contained in 0.2 ml of a 10^{-6} dilution of an 18 hour cul-

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* Supported by a grant from the Research Committee of the University of California, School of Medicine.

1. Jawetz, E., Gunnison, J. B., and Coleman, V. R., *Science*, 1950, v111, 254.

ture. In all experiments 0.2 ml of a 10^{-4} dilution of culture, about 100 LD_{50}, was injected intraperitoneally as the infecting dose. Commercial crystalline sodium penicillin G was freshly dissolved in saline prior to each experiment and suitably diluted. Chloramphenicol (Chloromycetin lot No. 129416†) was dissolved in saline in a concentration of 4 mg/ml and sterilized by Seitz filtration. Such stock solutions were kept at 4°C for 2-3 weeks without apparent deterioration. Final dilutions were prepared in saline just prior to each experiment. Mice were treated one hour after infection. Each drug was injected separately in a volume of 0.2 ml into the peritoneal cavity. Mice receiving no drug were injected with 0.2 ml saline. The 2 intraperitoneal injections took place within 1 minute. No further treatment was administered. The animals were observed for 10 days and a few of those dying in each group were autopsied to ascertain the cause of death. Heart blood cultured on blood agar plates in all instances yielded beta hemolytic streptococci in pure culture.

Results. Under the conditions of these experiments the estimated 50% curative dose of penicillin alone was 2-5 μg (0.1-0.25 mg/kg), and of Chloromycetin alone 400-600 μg (20-60 mg/kg). All untreated animals died within less than 3 days after infection. A basic observation on the joint effect of penicillin and chloramphenicol is shown in Fig. 1. Groups of 20 mice were infected in the standard manner with streptococcus C 203, and were treated with either penicillin 30 μg alone, or chloramphenicol 800 μg alone, or both drugs. Fig. 1 illustrates how either drug alone protected all but 20% of the animals, whereas the administration of both drugs resulted in a mortality of 60%. Thus apparently the two drugs interfered with each other's efficacy as therapeutic agents. This type of experiment was repeated many times, always with essentially the same results.

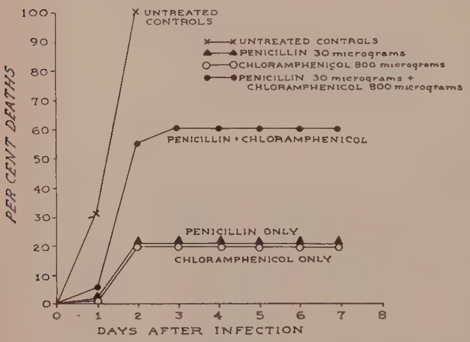

FIG. 1.

Joint action of penicillin (30 μg) with chloramphenicol (800 μg) on a streptococcal infection of mice.

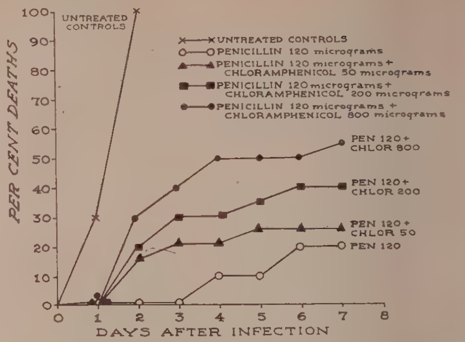

FIG. 2.

Joint action of a constant amount of penicillin (120 μg) with increasing amounts of chloramphenicol (50-800 μg) on a streptococcal infection of mice.

The quantitative relationships of this joint action of drugs was investigated by keeping the level of one agent constant and varying the other. The effect of increasing doses of chloramphenicol with a constant dose of penicillin is shown in Fig. 2. The more chloramphenicol is added to 120 μg of penicillin, the higher the death rate. With a smaller amount of penicillin, however, (*e.g.* 30 μg) this proportional increase was not observed. While 30 μg of penicillin alone protected all but 20% of the mice, the addition of 50, 200, or 800 μg of chloramphenicol resulted in 65, 60, and 60% deaths respectively.

When the dose of chloramphenicol was kept constant and the dose of penicillin varied, a different relationship was observed (Table I). Any combination of penicillin

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† Kindly supplied in generous amounts by Dr. G. Rieveschl, Parke, Davis and Co., Detroit, Mich.

TABLE I.
Joint Action of Penicillin with Chloramphenicol on a Streptococcal Infection of Mice.
20 g mice were given 100 LD_{50} of a culture of C 203 intraperitoneally. One hour later the drugs, or saline, were administered intraperitoneally, each in a volume of 0.2 ml, about 1 minute apart.

Streptococcus LD_{50}	Chloramphenicol, μg	Penicillin G, μg	Deaths/total in group	% deaths
100	800	0	9/26	34
100	0	6	3/16	19
100	0	120	2/26	8
100	800	6	11/16	68
100	800	30	12/20	60
100	800	120	18/36	50
100	50	6	11/16	68
100	50	30	13/20	65
100	50	120	13/35	34
0	800	120	0/16	0
100	0	0	26/26	100
10	0	0	12/12	100

with chloramphenicol resulted in a much larger number of deaths than the same dose of either drug alone, particularly penicillin alone. However, with any dose of chloramphenicol, within the experimental range, the more penicillin was administered, the lower was the rate of death.

Discussion. Chloramphenicol and penicillin, administered to uninfected mice in the doses used in these experiments, either alone, or together, never gave any evidence of toxicity. Consequently the higher death rate encountered with the joint use of both drugs could not be attributed to toxic depression of the host's defense mechanism, nor to direct toxic action of the drug. The evidence presented permits only the interpretation that in some manner penicillin and chloramphenicol interfere with each other's chemotherapeutic effect, in the experimental infection studied here.

This drug interference, or antagonism, might take place in one of 3 ways. (a) The two drugs could interact with one another in some physical or chemical process, so that the resulting complex was of lesser chemotherapeutic efficacy. (b) The 2 drugs might compete with one another for "receptors" on the susceptible bacterial cells. It is conceivable that through mass action the less effective drug might thus interfere with the more potent one. (c) Possibly one drug might so change developmental characteristics or properties of the infecting microorganisms that their susceptibility to other agents is greatly diminished. At present no definite choice can be made among these possible mechanisms. However, experiments are being designed to clarify this issue. The data at present available are compatible with the hypothesis that in some way chloramphenicol quantitatively interferes with penicillin effect.

Antagonistic influences of one antibiotic agent upon another have been observed by others(2) using a different laboratory model. In the experiments of Price *et al.*(2) the differences between single and multiple drug action appeared relatively small, and a shift from apparent synergism to antagonism was observed with 4- to 10-fold changes in drug concentrations. No immediate comparison of their data with ours is therefore possible. The clear-cut antagonistic action between chloramphenicol and penicillin demonstrated here is of interest in several regards. It draws attention to the possibility of interfering with, rather than potentiating, the action of one chemotherapeutic agent by administering it together with others. It has been increasingly popular medical practice to administer several chemotherapeutic agents simultaneously to patients, in the expecta-

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2. Price, C. W., *et al. Am. J. Publ. Health,* 1949, v39, 340.

tion that such therapy would more rapidly and effectively terminate an infectious process. Such multiple chemotherapy has often been employed in the absence of accurate bacteriological diagnosis. In some instances it has been conclusively shown that several antimicrobial drugs administered together are more effective than one alone. The material presented here strongly suggests that the opposite may sometimes be the case. If antagonistic effects between antibiotic agents play any role in clinical practice it may become necessary for physicians to use such drugs only for specific indications, and abandon "shotgun therapy".

The observed antagonism between antibiotic agents may also provide a useful laboratory tool for the investigation of antibiotic action, provided it can be shown that it is not due to a simple chemical inactivation of one drug by another, but rather to an interference between the drugs at their site of action.

Addendum. Since this paper was submitted for publication additional experiments have been carried out with a separation of the two antibiotic agents in space and in time. Similar antagonistic results were obtained when the two drugs were injected subcutaneously at widely separated sites, or when they were given in different sites one hour apart.

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Resistance to Epinephrine Stress in the Dog.* (17821)

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Current concepts of the physiologic activation of the pituitary-adrenal mechanism suggest that epinephrine is the humoral agent responsible for the release of ACTH from the anterior pituitary(1,2). It is not known whether epinephrine acts directly on the anterior pituitary or by stimulating the hypothalamus(3,4). The hypothalamus in turn may secrete an adrenergic substance(5) into the hypothalamo-hypophysial portal system and thereby stimulate the anterior pituitary to release ACTH. Other evidence suggests that epinephrine released as a result of sympathico-adrenal discharge may act peripherally to increase the tissue utilization of 11-oxysteroids, leading to lowered plasma levels and activation of the anterior pituitary to release ACTH(6). The site of action of epinephrine is further disputed by conflicting reports in the hypophysectomized rat wherein epinephrine effects a lymphopenia in one laboratory(7) whereas another group reports that epinephrine effects no increase in adrenal cortical size or lipid content in the absence of the pituitary(8). It has been suggested that in man, large doses of epinephrine may act directly on the adrenals(2). Epinephrine is a key hormone in present thinking on pituitary-adrenal relationships. Pharmacologically speaking, it is believed that no true tolerance to epinephrine exists. This concept must be qualified. Continuous or repetitive administration of epinephrine fails to produce continuous hyperglycemia or gly-

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* Aided by grants from the Office of the Surgeon General, Department of the Army, and The Chicago Heart Assn.

† Public Health Service Research Fellow of the National Heart Institute.

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cosuria(9,10). This tachyphylactic type of response is believed to be due to depletion of liver glycogen stores(11). Repeated daily subcutaneous injections of epinephrine to rats has been reported to produce no significant rise in blood pressure(12). Another example of epinephrine tolerance can be demonstrated with sub-lethal doses of the drug(13). Following administration of sub-lethal doses of epinephrine to dogs(14) and rabbits(15), lethal doses will no longer produce death. Clinically it is known that some asthmatic patients develop a refractoriness to epinephrine. The concept of epinephrine-histamine "see-saw" has been invoked to explain this phenomenon(16).

It has been demonstrated that repeated exposure to a single stress results in the development of a stage of resistance to that stress. In this stage of resistance, the animal is still sensitive to a different stress(17). Refractoriness of the pituitary-adrenal mechanism to epinephrine as a stress has been implied in the human(2). In view of the importance of epinephrine as a possible physiologic activator of the pituitary-adrenal mechanism, it was of interest to determine whether a "resistance" could be experimentally produced to epinephrine. It has been previously reported that repetitive daily administration of epinephrine to the unanesthetized dog resulted in a diminished eosinopenic response(18). The present paper presents further data on this interesting phenomenon.

Methods. One of the most sensitive and reliable criteria of the release of 11-17-oxysteroids from a functioning adrenal cortex is the depression of the circulating eosinophils in the blood(19). Thus the eosinophil response was chosen as a criterion of adrenal cortical discharge. The administration of epinephrine has been shown to effect an eosinopenia in man(2,19) and in the dog (4,18,20).

Eosinophil counts were made by a modification of the acetone-eosin method of Dunger (21). Four Spencer Bright-Line chambers were counted in each determination. The average of 4 chambers was multiplied by a factor of 11.1 to give the cells/mm^3 of blood. Two separate control counts were done at the start of each experiment, if these failed to check within 10% of each other, a third count was made and the average of the 3 taken as the base line. Eosinophil counts were made at the conclusion of the epinephrine infusion and thereafter at hourly intervals for 5 hours. Repetitive, hourly, eosinophil counts on unanesthetized trained dogs under controlled conditions in our laboratory yielded a mean adjusted coefficient of variation (twice the usual coeff. of var.) of +11.1%(22). The mean of a given dog's control counts plus or minus the adjusted coefficient of variation would approximately include 95% of the dog's control counts. All experiments were conducted on trained unanesthetized female dogs weighing between 15 and 25 kg. The hyperglycemic effect of epinephrine was used as an additional criterion of pharmacologic activity of the drug. Serial plasma glucose determinations were made by Nelson's method(23) using a

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TABLE I.
Development of Epinephrine Resistance in the Dog.

Dog	Max. depression of eosinophil count (%)† Day of experiment							
	1	2	3	4	5	6	7	8
A. 5 μg/kg/min. Dose of Epinephrine.*								
G.	57	18	6	(+1)	(+12)	—	—	—
Q.	67	41	37	23	8	—	—	—
C.	47	6	5	7	(+6)	16	+9	—
T.	63	64	50	27	—	32	—	—
S.	32	18	23	47	—	—	82	16
H.	40	—	—	29	40	30	13	18
B. 10 μg/kg/min. Dose of Epinephrine.*								
G.	66	18	24	12	—			
G.	42	32	58	52	—	—		
H.	50	—	95	—	—	—		
C.	44	7	8	—	12	—		
Q.	71	62	69	44	55	—		

* Infused for 60 minutes.
† Calc. as 100 —% of pre-treatment control counts.

Somogyi filtrate(24). Parke-Davis adrenalin hydrochloride‡ was used as a source of epinephrine. In preliminary experiments it was found that the dog was strikingly more resistant than man in the terms of the doses of epinephrine required to effect eosinopenic reactions. Single doses of 5-30 μg/kg injected intravenously produced marked circulatory and hyperglycemic responses but no eosinopenia. The continuous intravenous infusion of 5 μg/kg/min for 60 minutes depressed the eosinophile counts in excess of 30% in 16 out of 20 experiments in 11 dogs. In 5 experiments on 4 dogs, an infusion of 10 μg/kg/min for 60 minutes produced an eosinopenic response in excess of 30% in all runs. Therefore, epinephrine was administered daily in doses of 5-10 μg/kg/min for 60 minutes in an attempt to produce a state of resistance. A dose of 20 μg/kg/min was fatal to one dog in which it was tried.

Animals that developed a resistance to epinephrine were exposed to other different stresses in an attempt to elucidate the mechanism of epinephrine refractoriness. Hyperglycemia as a stress was produced by the intravenous infusion of 15% glucose in water at the rate of 8 cc/min for up to 5 hours. This procedure effects marked eosinopenia in the dog(22). Histamine§ was administered intravenously at a dose of 5 μg/kg/min for 60 minutes. This dose of histamine results in both an immediate and delayed eosinopenic reaction in the dog(25). In addition, exposure to cold (3°C for 1 hour) or starvation were employed to activate the pituitary-adrenal mechanism. ACTH‖ equivalent to 50 mg of Armour Standard was injected intramuscularly to determine possible "exhaustion" of the adrenal cortex following repeated exposure to epinephrine stress.

Results. The data in Table IA represent maximum depressions of the eosinophil count occurring in a series of 6 dogs following the repeated administration of epinephrine at a dose of 5 μg/kg/min. In 3 out of 6 dogs a definite resistance to epinephrine developed. In the remaining 3 dogs the eosinopenic response was appreciably diminished.

Table IB summarizes the eosinopenic responses to the 10 μg/kg/min dosage level in 4 dogs. In the dog G., one experiment resulted in development of resistance whereas a repeat run was negative. Only 1 of the re-

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23. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

24. Somogyi, M., *J. Biol. Chem.*, 1945, v160, 69.

‡ Obtained through the kindness of Dr. E. A. Sharp of Park-Davis and Co.

§ Obtained through the kindness of Dr. M. J. Schiffrin of Hoffmann-LaRoche, Inc.

25. Last, J. H., Jordan, P., Pitesky, I., and Bond, E., *Science*, in press.

‖ We are indebted to Dr. Edwin Hays of Armour Laboratories for a generous supply of ACTH.

TABLE II.
Hyperglycemic Responses to Repeated Administration of Epinephrine in the Dog.

Dog	Day of epinephrine infusion	Max. decrease in eosinophils (%)	Blood sugar (mg%) Time after onset of infusion (min.)				
			Control	60	120	180	240
A. 5 μg/kg/min. Dose of Epinephrine.							
Q.	1	67	69	325	63	—	82
	3	37	78	339	—	—	78
C.	1	47	58	165	112	92	89
	3	5	82	275	148	95	74
	5	+6	90	294	170	112	96
T.	1	63	51	175	50	49	85
	3	50	53	265	99	53	66
	6	32	55	168	—	70	58
S.	1	32	74	260	75	49	69
	3	23	50	228	81	—	75
	7	82	50	114	98	84	81
H.	1	40	62	234	86	80	69
	4	29	65	173	79	82	—
	6	30	50	276	98	107	113
	8	8	85	260	106	121	128
B. 10 μg/kg/min. Dose of Epinephrine.							
G.	1	66	69	291	131	82	59
	3	24	75	341	166	96	64
G.	1	42	82	359	140	83	79
	3	58	86	205	185	114	103
C.	3	8	75	383	99	71	74
Q.	1	71	90	245	—	184	101
	4	44	73	285	158	114	75

maining 3 dogs demonstrated the epinephrine resistance phenomenon. There was no demonstrable cumulative eosinopenic effect of the daily administration of epinephrine at the two dosages used. The control pre-infusion eosinophil counts on successive days were comparable in magnitude to those eosinophil levels occurring prior to any epinephrine therapy. The hyperglycemic responses to the epinephrine infusions are presented in Table II. The pharmacologic effectiveness of the repetitive epinephrine infusions is apparent in the hyperglycemic responses. Hyperglycemia was produced by infusions of epinephrine that failed to produce marked eosinopenia. Dogs in the epinephrine refractory state were exposed to secondary stresses, larger doses of epinephrine, or ACTH. The eosinophil and hyperglycemic responses to these stresses are presented in Tables III, IV, and V. From Tables III and IV it can be seen that the resistant state is probably not due to a depletion of endogenous ACTH since secondary stresses effected eosinopenic responses. Further evidence in this direction is presented in Table IV where it can be seen that an increased dose of epinephrine effected an eosinopenic response in an animal resistant to a dose of 5 μg/kg/min. Had endogenous ACTH supplies been depleted, neither the higher dose of epinephrine nor the subsequent glucose stress should have produced eosinopenia.

The data in Table V, although somewhat extraneous to the thesis of this paper, are presented for their theoretical implications. Initially an epinephrine dose of 5.0 μg/kg/min produced a questionable eosinopenic response. The animal was then starved for 2 weeks with the production of a state of resistance to starvation as a primary stress. In keeping with present concepts

TABLE III.
Effects of Stresses Superimposed on a State of Epinephrine Resistance.
Dog Co, ♀, wt 16 kg.

Date	Epinephrine stress,* µg/kg/min.	Second stress	Max. decr. in eosinophils %	Blood sugar (mg%) Time after onset of infusion (min.)				
				Control	60	120	180	240
6/13/49	5	—	47	57	165	112	92	89
14	5	—	6.5	—	—	—	—	—
15	5	—	5.5	84	275	148	95	74
16								
A.M.	5	—	7.7	—	—	—	—	—
P.M.	—	Exposure to cold†	20	—	—	—	—	—
17								
A.M.	5	—	(+0.9)	91	294	170	112	—
P.M.	—	ACTH‡	40.5	—	—	—	—	—
18	5	—	15.6	—	—	—	—	—
20	5	—	(+9.6)	—	—	—	—	—
21	—	Hyperglycemia§	54	105¶	1240¶	895¶	800¶	800¶
22								
A.M.	5	—	18.5	—	—	—	—	—
P.M.	—	Histamine‖	34.8	—	—	—	—	—

* Epinephrine infused for 60 min.
† Exposure to 3°C for 1 hr.
‡ 50 mg of Armour Standard inj. i.m.
§ 15% glucose infused i.v. at 8 cc/min. for 5 hrs.
‖ 5 µg/kg/min. of histamine (calc. as free base) infused i.v. for 60 min.
¶ Arterial blood samples.

TABLE IV.
Effects of Increasing the Epinephrine Dose on a Pre-existing State of Epinephrine Resistance.
Dog Gi, ♀, wt 18 kg.

Date	Epinephrine stress,* µg/kg/min.	Second stress µg/kg/min.*	Max. decr. in eosinophils %	Blood sugar (mg%) Time after onset of infusion (min.)				
				Control	60	120	180	240
7/12/49	5	—	57.5	—	—	—	—	—
13	5	—	17.6	86	220	55	67	68
14	5	—	5.8	—	—	—	—	—
15	5	—	(+0.8)	—	—	—	—	—
16	5	—	(+11.9)	—	—	—	—	—
17	—	10	64.5	67	291	131	82	59
19	—	10	19.2	—	—	—	—	—
20	—	10	33.6	75	341	165	96	64
21	—	10	3.7	—	—	—	—	—
22	—	Hyperglycemia†	58.0	69	752	935	1102	1022

* Epinephrine infused for 60 min.
† 15% glucose infused i.v. at 8 cc/min. for 5 hrs.

(17), the animal was markedly sensitive to a second stress, epinephrine, which was administered at a much lower dosage than initially. Of great interest was the ensuing phenomenon of the development of a refractory state to this second stress. That the adrenal cortex was not exhausted even in the face of these two stresses, is adequately demonstrated by the eosinopenic reaction to ACTH. That the endogenous supply of ACTH was probably not exhausted was demonstrated by the eosinopenic response to 5.0 µg/kg/min of epinephrine, although the magnitude of the response is admittedly moderate.

Discussion. In terms of one criterion of the endogenous release of 11-17-oxysteroids namely the eosinopenic response, the data indicate that the repetitive daily administration of epinephrine at a dose of 5.0 µg/kg/min infused for 60 minutes, results in a state of relative resistance or refractor-

TABLE V.
Epinephrine Resistance Superimposed on State of Resistance to Starvation Stress.
Dog Tr., ♀, wt 16 kg.

Date	Primary stress	Secondary stress	Max. decr. in eosinophils %	Blood sugar (mg%) Time after onset of infusion (min.)				
				Control	60	120	180	240
5/16/49	5.0 μg/kg/min.*	—	20	—	—	—	—	—
18	Starvation—through 6/4/49 (Water fed *ad lib.*)							
31	"	.5 μg/kg/min.*	95.5	60	173	160	155	137
6/ 1	"	.5 "	32.	—	—	—	—	—
2	"	.5 "	46.	58	135	108	98	81
3								
A.M.		.5 "	9	—	—	—	—	—
P.M.		ACTH†	64	—	—	—	—	—
6/4	"	5.0 μg/kg/min.*	26	—	—	—	—	—

* Epinephrine infused for 60 min.
† 50 mg of Armour Standard inj. i.m.

iness. During this state, other pharmacologic effects of the drug such as the mobilization of glycogen and the production of hyperglycemia are not diminished. Higher dosages of epinephrine (10 μg/kg/min) fail to produce the same degree of resistance although a diminished eosinopenic response was noted in some animals. Neither dosage levels of epinephrine seemed to deplete liver glycogen stores under the conditions of these experiments. Resistance to epinephrine as a stress can be explained by several mechanisms. Depletion of endogenous ACTH would be the simplest explanation, however, the eosinopenic response to secondary stresses would seem to speak against this hypothesis. Exhaustion of the adrenal cortex might also explain the refractory state, but again this is unlikely in view of the marked eosinopenic response to exogenous ACTH. Admittedly the dose of ACTH is very large, however the clinical reactions of the dogs receiving both dosage levels of epinephrine were also striking in terms of the severity of the stress. The animals showed profound dyspnea, cardiac arrhythmias, frequently vomited and in some instances went on to a bloody diarrhea. One would expect that toxic stress of this severity would liberate maximal quantities of endogenous ACTH if such stress acted on the pituitary.

A third explanation of the epinephrine resistance state might be that with adaptation to epinephrine as a stress, the tissue utilization of corticoids decreases. With the decreased utilization of corticoids, the stimulus for ACTH production is removed and no eosinopenia would result. This theory has been substantiated by experimental work in the rat(6) and would satisfactorily explain the epinephrine resistant state as well as the eosinopenic responses to secondary stresses and exogenous ACTH.

The experimental demonstration of an epinephrine resistant state does not exclude the possibility that stress effects the release of epinephrine which acts as the continuous physiologic activator of the pituitary-adrenal mechanism, but it makes such a hypothesis difficult to accept. Recent work in the human substantiates this concept that the sustained secretion of ACTH may not be due to the continuous action of epinephrine(2). It has been stated that epinephrine in large doses may act directly on the adrenal cortex. Under the conditions of these experiments, the lack of eosinopenia could be explained if epinephrine acted directly on the adrenal cortex, which then became refractory to this stress. The adrenal cortex was sensitive to endogenous ACTH since other stresses effected eosinopenia during epinephrine. Thus in addition to refractoriness of the adrenal gland to epinephrine, one must postulate that the pituitary is equally refractory so as not to release ACTH, to which the adrenal is sensitive, during the epinephrine resistant stage. It would therefore appear that a direct action of epinephrine on the adrenal cortex is problematic.

Summary. 1. The repetitive daily administration of epinephrine in the dog results in a progressively diminished eosinopenic response.

2. During the state of epinephrine resistance, there is neither a depletion of endogenous ACTH nor an exhaustion of the adrenal cortex.

3. Adaptation of the organism to epinephrine stress with decreased tissue utilization of corticoids seems to most satisfactorily explain the phenomenon of epinephrine resistance at the present time.

4. Demonstration of resistance to epinephrine speaks against epinephrine as the only physiological stimulus for the continuous secretion of ACTH.

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Inhibition of Vascularization of the Rabbit Cornea by Local Application of Cortisone.* (17822)

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It has become apparent that one of the most striking properties of cortisone is its selective depression of certain mesenchymal cells, as evidenced experimentally in the effect on mouse lymphosarcoma(1) and on wound healing(2). Presumably, the antirheumatic effect of cortisone is likewise due to the selective suppression of cells of mesenchymal origin, involved in an as yet undetermined mechanism in the production of rheumatic diseases. It was thought of interest to investigate whether capillary endothelial cells were among those influenced by cortisone. The vascularization of the cornea appeared to be most suitable for the study of this effect, especially since cortisone might be expected to exert its action on capillary growth when applied locally. Such local application would allow use of the fellow eye as a control. The vascular responses to corneal injury are well known, having been investigated thoroughly by several authors (3,4).

As will be shown by the data here presented, the subconjunctival injection of cortisone did indeed inhibit the growth of capillaries into a chemical burn of the cornea to a high degree.

Method. Three groups containing 6 albino rabbits each were used. The first group sustained corneal burns with 0.2 N alkali, and the second and third groups received burns with 0.5 N alkali. The control eyes in the first and second groups were given no subconjunctival injection, while the third group's control eyes received a suspension of cholesterol in the same medium as the cortisone suspension. The experiments were carried out in the following manner: under topical nupercaine anesthesia, an injection of 0.03 cc NaOH in the concentrations mentioned above was made into the superficial corneal layers half way between the apex of the cornea and the superior limbus with a 30 gauge needle. This was done on both eyes of all animals. A burn about 3 mm in diameter resulted when the weaker solution

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* The work was supported in part by grants from the Helen Hay Whitney Fund and the United States Public Health Service.

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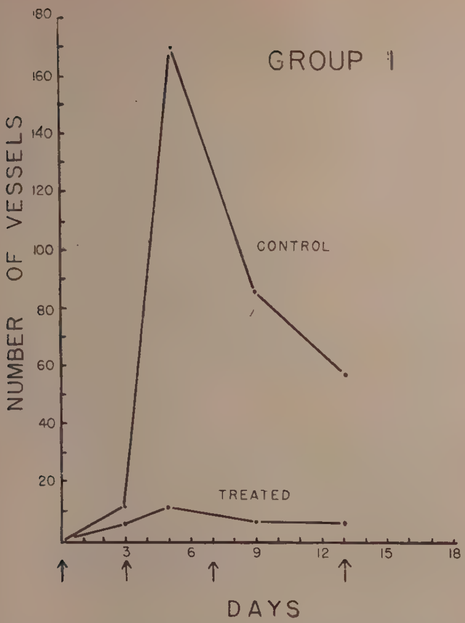

Fig. 1.

Comparison of number of corneal vessels in 6 control (right) and 6 treated (left) eyes following intracorneal injection of 0.2N NaOH. The arrows indicate injection of 0.05 ml (1.25 mg) of cortisone acetate.

was used. In one eye of each animal a saline suspension of cortisone acetate (Merck) containing 1.25 mg in 0.05 cc was injected subconjunctivally at the point nearest the burn. The control eyes of the third group received a similar injection of cholesterol. Penicillin ointment was used in the conjunctival sacs on the operative day. Reinjections of cortisone were done on the third and seventh days in Group I, and on the third, sixth, and tenth days in Group III. Cholesterol injections of the control eyes in Group III were carried out at the same time cortisone was reinjected. In Group II on the seventh day cortisone was injected subconjunctivally in the control eyes. All eyes were examined daily with a loupe during the florid phase, and at longer intervals thereafter as the activity subsided.

Results. The results are expressed as the number of vessels invading the cornea. In the graph, it should be noted that the peaks occurred at the same time, and that disappearance of vessels was rapid on the control side (Fig. 1). The graph for Group II shows simultaneous peaks on the fourth day, but the treated eyes, receiving no new cortisone when the original injection was exhausted, reached a new peak on the seventh day. At this point cortisone was injected in the control eyes, but not in the treated, and a precipitous decline in the number of vessels in the control eyes occurred. Whether this can be credited to cortisone is doubtful, in view of a similar decline in the number of vessels in control eyes of Group I in the absence of cortisone influence (Fig. 2). Group III shows a retardation of new vessel formation due to the severity of the burns. Otherwise, the shape of the graph is in conformity with that on the previous group (Fig. 3).

The length of the newly-formed vessels, while greater on the control than on the treated side in all groups, did not seem significantly different.

Slit lamp examination of treated and con-

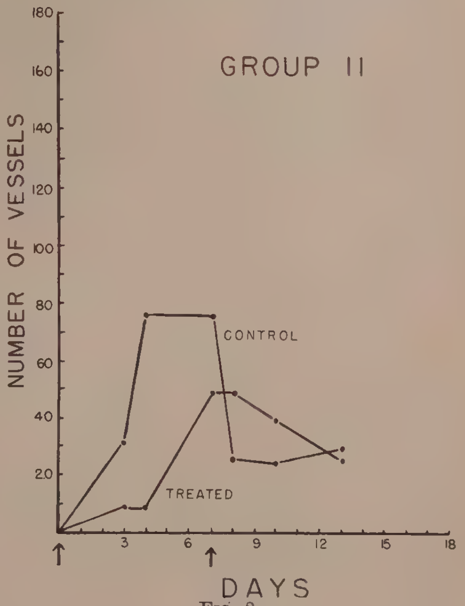

Fig. 2.

Both eyes treated as in Fig. 1, except that 0.5N NaOH was injected. The treated eyes were injected with cortisone only on the first day. The control eyes were injected with cortisone on the seventh day.

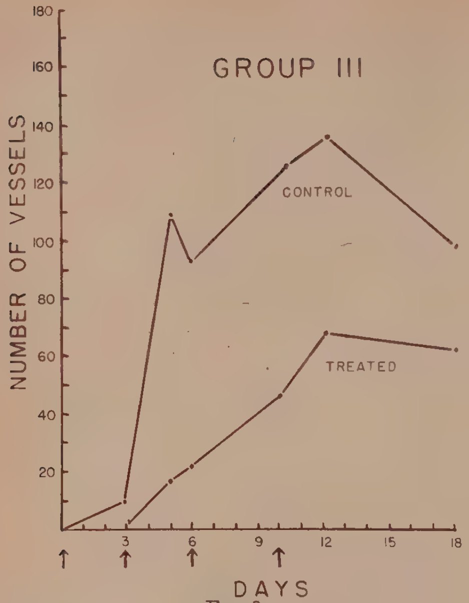

Fig. 3.

Same as Fig. 1, except that 0.5N NaOH was used.

trol eyes showed all the vessels to be superficial, and revealed no gross difference in caliber or branching.

Discussion. The experiments reported here appear to demonstrate the depression of capillary growth by the local application of cortisone. The mechanism of this action is unknown. Two possible mechanisms suggest themselves as the mode of action of cortisone: (1) the blocking of the chemotactic influence on the limbus capillaries exerted by the injured corneal tissue, and (2) the prevention of the response of the endothelial cells to a chemotactic stimulus by inhibition of processes essential for capillary proliferation. Vascularization of the cornea has been suggested to be initiated by the enzymatic hydrolysis of the mucopolysaccharide of the ground substance of the cornea(5). Cortisone has been claimed to be an inhibitor of the action of hyaluronidase both on dermal spreading in animals(6) and in man(7) and on the permeability of the synovial membrane(8). Corneal vascularization appears to be especially suited to submit these hypotheses to experimental proof.

Summary. The subconjunctival injection of cortisone inhibits markedly the vascularization of the cornea caused by intracorneal injection of alkali.

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Antihistamine Therapy in Experimental Shock.* (17823)

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Among the theories proposed to explain the cause of secondary shock there have been many suggestions that histamine, "H substance" and similar agents, released by injured tissue, account for the shock symptoms. Repeated injections of histamine have been reported by Dale and Laidlaw(1) to induce fluid loss and cardiovascular changes typical of secondary shock. In shock from burns and occlusion tourniquets loss of fluid from the blood has been considered an important contributing factor to cardiovascular failure. The possibility that such fluid shifts might be reduced by the use of antihistamine

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* Supported in part by U. S. Public Health Grant.

1. Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1919, v52, 355.

TABLE I.
Tourniquet Shock Mortality in Treated and Control Rats.

Treatment	No. of animals	% mortality 24 hr	Temp. °C
Benadryl, 20 mg/kg	62	90	28.5-32.5
Controls—Saline	83	76	,, ,,
Thephorin, 25 mg/kg	72	58	
Controls—Saline	76	41	24-28
Thephorin, 50 mg/kg	71	93	,,
Controls—Saline	67	66	,,

drugs has suggested itself to several experimenters. Halpern(2) reported that passage of dye from the peritoneal cavity was reduced, and pumonary edema from epinephrine administration was prevented by dimethylamino - 2 - propyl - L - phenolthiazine (Phenergan). Lowy and Code(3) applied antihistaminics locally in man and found no influence on flares produced by small skin burns, but found flares induced by local freezing could be inhibited by the drugs. Jourdan and Chatonnet(4) found no beneficial results when shock induced by muscle trauma was treated with N-dimethylamino ethyl-N-benzylaniline (Antergan). In view of these conflicting reports, a series of experiments were undertaken to test the effects of antihistaminics on survival and fluid loss in experimental shock.

Methods. Rats anesthetized with 32.5 mg/kg sodium pentobarbital were shocked by application of rubber bands to the hind legs for 3 hours and 40 minutes. According to Shipley, Meyer and McShan(5) this procedure results in 47% mortality, but our rate (41-76%) was somewhat higher, which we have ascribed to the high room temperatures prevailing during the summer when much of the work was done. Two antihistaminics were used, Beta-dimethylaminoethylbenzhydryl ether hydrochloride (Benadryl)[†] and 2-methyl-9-phenyl-2,3,4,9-tetrahydro-1-pyridindene (Thephorin)[‡]. Benadryl was given intraperitoneally 20 mg/kg in one dose about 15 minutes before release of the tourniquet, or in two doses, with the second about 4 hours after release. Thephorin was given in doses of 25 or 50 mg/kg, either as a single intraperitoneal injection, or in 2 doses, the first given about 15 minutes before application, and the second before release of the tourniquets.

Dogs deeply anesthetized with the dose of pentobarbital given above, plus 5 mg/kg morphine sulfate, were shocked by exposing the hind quarters to water at 90°C for 5 seconds(6). Before shock, 20 mg/kg Benadryl i.m. was given, followed after shocking by 5 mg/kg every 3 hours for 24 hours. In a second series, the shock therapy consisted of 40 mg% Benadryl in saline administered intravenously at a rate of 100 cc/kg/24 hours. Control animals were given saline instead of the drug in both groups. Hematocrit readings, and blood and serum specific gravity determinations by the copper sulfate method were made before shock, and at 3-hour intervals thereafter. Blood pressure was recorded from the carotid artery by a mercury manometer.

Results. The 24-hour mortality rate for rats shown in Table I indicates that neither of the 2 antihistaminics used were of benefit in tourniquet shock. Large doses, in fact, decreased survival noticeably.

Table II summarizes the results of burn shock treatment in dogs. No marked decrease in mortality resulted from Benadryl

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2. Halpern, B. N., *Am. J. Physiol.*, 1948, v155, 442.

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5. Shipley, E. G., Meyer, R. K., and McShan, W. H., Proc. Soc. Exp. Biol. and Med., 1945, v60, 340.

[†] Supplied by Parke, Davis and Co., Detroit, Mich.

[‡] Supplied by Hoffmann-LaRoche, Inc., Nutley, N. J.

6. Elman, R., and Brown, F. C., *War Med.*, 1943, v3, 477.

TABLE II.
Burn Shock Mortality in Treated and Control Dogs.

Treatment	No. of animals	% mortality 24 hr	Avg hr survival
Benadryl i.m.	28	89	11.5
Controls—Saline i.m.	26	85	12.9
Benadryl + saline i.v.	11	64	19.4
Control—Saline i.v.	11	73	14.4

therapy, although there may be some suggestion that saline infusion plus this drug aided survival. Hematocrit, and blood and serum specific gravity changes did not vary consistently between treated and control groups. Blood pressure readings in the treated group tended to be slightly above those of the controls throughout the survival period, but this did not appear to be of significant magnitude.

Discussion. The results above indicate that the 2 antihistaminics used were without benefit, or somewhat detrimental, to animals in burn and tourniquet shock. Page and Green(7) found that large doses of Benadryl caused vascular refractoriness in the dog, with the result that larger than normal amounts of saline could be infused into the animal. This was ascribed to increased capacity of the vascular bed, or to increased permeability of the capillaries. Our results in the shocked dog do not suggest this, since the changes in blood pressure and hemoconcentration were not consistently different in the control and treated animals. Possibly the larger doses of Benadryl used by Page and Green account for the refractory state of the vascular bed in their animals.

Summary. The administration of Benadryl or Thephorin to rats subjected to tourniquet shock tended to increase mortality if given in relatively large doses. Dogs in burn shock treated with Benadryl did not differ significantly from untreated controls with respect to mortality, blood pressure, hematocrit, and blood and serum specific gravity.

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A Rapid Method for Determination of the Aureomycin Concentration of the Blood. (17824)

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The need for a speedy report of the blood concentration is particularly urgent with aureomycin therapy. Nausea, vomiting and diarrhea are not uncommon with this drug, and it is often difficult to decide whether or not the administered drug has been adsorbed and to what extent. This need is further emphasized by the increasing use of such adjuvants as aluminum hydroxide gels to counteract the toxic symptoms. Waisbren and Hueckel(1) have shown that when aluminum hydroxide gel is administered with aureomycin, a lowered level of aureomycin in the blood results. A method of assay which can be completed and reported within 5 hours after the specimen is received in the laboratory, no matter what time of day or night, is the subject of this report. The procedure is a tube dilution method, employing *Proteus vulgaris* OX-19 as the standard organism and a special urea-phenol red broth as the medium. The principle of the test is based upon the bacteriostatic action of the antibiotic on the test strain and the inhibition of the rapid

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1. Waisbren, B. A., and Hueckel, J. S., Proc. Soc. Exp. Biol. and Med., 1950, v73, 73.

TABLE I.

					ml				Controls		
Aureomycin Standard											
Aureomycin in Normal Human Serum (2 μg/ml)					.5	.4	.3	NHS*	0	1	0
Urea Medium					.5	.6	.7		1	0	1
Standard Inoculum					1.	1.	1.		1	1	0
Serum Sample.											
Serum	1.	.8	.6	.5	.4	.35	.3	.25	.2	.15	.1
Urea Medium	0	.2	.4	.5	.6	.65	.7	.75	.8	.85	.9
Standard Inoculum	1.	1.	1.	1.	1.	1.	1.	1.	1.	1.	1.

* Normal Human Serum.

reaction and color change when it splits urea to ammonia. No other organisms are capable of producing the color change within the time limit of the test, thus obviating the need for sterile technic. Since the incubation period at 37°C is approximately 4 hours, deterioration of aureomycin is minimal. Finally, the endpoints are indicated by color changes and are easily read.

Method. Blood for assay is collected in a dry, clean centrifuge tube and the serum is separated in the usual fashion. Care must be exercised to prevent hemolysis as the test is dependent upon the development of a red color. The test employs 2 ml amounts and is performed in unplugged 12 x 100 mm tubes. Clean, but not sterile glassware, pipettes, etc. are used. The medium described by Stuart, Van Stratum and Rustigan (2), modified by the addition of 1% Bacto-Peptone as added nutritive material in order to accelerate the growth of the test organism, is employed. It is prepared as follows: To 380 ml of distilled water are added 3.64 g KH_2PO_4, 3.8 g Na_2HPO_4, 8.0 g Urea C. P., 40 mg Yeast Extract Difco, 4.0 g Bacto-Peptone Difco, and 20 ml Phenol Red (0.02%). The pH is usually 6.5-6.6, and is adjusted by the addition of 10% NaOH to a final pH of 6.8, at which point the medium is colored deep amber. It is stored in the refrigerator without being sterilized. The amount necessary to perform one or a series of tests is withdrawn as needed. The American Type Culture Collection strain of *Proteus vulgaris* OX-19 is the standard organism. Plain fresh meat extract broth is inoculated with this organism and incubated for 4 hours at 37°C and then diluted 1:5 with the same broth. The diluted broth is distributed into small tubes in approximately one milliliter amounts and stored in this manner in the refrigerator as individual stock cultures. These can be kept and used for 2 weeks. When indicated, a tube is removed and used to prepare the standard inoculum, which is a 1:100 dilution of the stock culture in the urea medium. Preliminary incubation of the standard inoculum is unnecessary, hence the test can be started at any hour of the day or night.

The aureomycin standard, 2.0 μg of aureomycin per ml in normal human serum, is freshly prepared just prior to the assay from a concentrated standard maintained in the freezing compartment of the refrigerator. Pooled excess serum from specimens sent for serological examination is readily available in the laboratory and can be used for the preparation of the aureomycin standard and for the controls.

Two parallel series of tubes are set up as shown in Table I.

Both series of tubes are incubated at 37°C for 4 hours at which time the inoculated controls usually have a definite red color. Occasionally, 4½-5 hours are required to complete the reaction. The endpoint, indicating complete inhibition of the test organism by the antibiotic, is the first tube in each series in which the amber color fails to show any change to red. The serum level is calculated by dividing the amount of standard antibiotic required to inhibit the standard inoculum by the amount of serum required to inhibit the same inoculum. If inhibition takes

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2. Stuart, C. A., Van Stratum, E., and Rustigan, R., *J. Bact.*, 1945, v49, 437.

place throughout the entire serum series, the serum is diluted 1:10 in the urea medium and the test is then repeated with diluted serum. The level is calculated in the same manner, multiplied by the dilution factor.

Experimental results. It was found that the standard inoculum was consistently inhibited by 0.8 μg of the standard aureomycin. Thus the minimum assayable concentration of aureomycin in the serum is 0.8 μg per ml of serum. Although this is not as sensitive as methods in current use, it is adequate for clinical purposes, since the minimum detectable level is well within the range of blood concentrations regularly found with the doses commonly employed.

To test the accuracy of this procedure, 36 samples of normal human serum were fortified with known concentrations of aureomycin just prior to testing and then assayed. The results of the assay proved accurate in 28 out of 36 tests, or 77.8%. In the remaining tests there was a discrepancy between the prepared and calculated values, but in no instance was there any error greater than one tube, except in one, which was two tubes under. Considering the experimental error arising from the preparation of the test specimen as well as the performance of the assay, a high correlation was found between the concentration of the prepared specimens and the result of assay.

Summary. A rapid method for the determination of the aureomycin concentration of the blood is described. Sterile specimens and sterile technic are not required. The procedure is based upon the inhibition of the test organism, *Proteus vulgaris* OX-19 by the antibiotic, thereby preventing the reaction and color change produced in the urea-phenol red medium when urea is split to ammonia by the test strain. The minimum assayable level is 0.8 μg per ml of serum. A high degree of correlation was found between the actual value and the results of assays with 36 prepared specimens.

I am greatly indebted to Miss Joan Greenwald for her capable technical assistance.

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Characterization and Separation of an Inhibitor of Viral Hemagglutination Present in Urine. (17825)

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Hemagglutination caused by influenza, mumps and Newcastle disease viruses is inhibited by a number of biological materials which possess such activity in varying degree(1-3). The various inhibitors have certain properties in common: when tested with appropriately heated viruses, they are active in high titer, but their activity is destroyed by the unheated viruses; it is also destroyed by cholera vibrio extract, crystalline trypsin, or periodic acid. The inhibitors are substances of high molecular weight and are thought to be mucopolysaccharides or mucoproteins. The interaction between inhibitors and viruses has been studied in considerable detail by numerous workers(4-7), but purification of the inhibitors has met with difficulties which have not been completely overcome(7-14). Among the most

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7. Hardy, P. H., Jr., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, v88, 463.

TABLE I.
Inhibition of Hemagglutination by Different Viruses with Normal Human Urine.

Virus		Urine* inhibition titer	Virus		Urine* inhibition titer
	Units, final		Heated, °C	Units, final	
PR8	3	16	56 30′	2	1024
FM1	2	8	” ”	2	512
Lee	2	4	” ”	2	512
Swine	2	1024	52 ”	2	64000
Mumps	2	128	46 20′	2	128
NDV	3	4	50 ”	2	32

* A single specimen of urine from a normal adult male was employed. It was neither dialyzed nor heated.

active of the viral inhibitors is a purified erythrocyte extract which, in an amount of 0.1 μg, inhibits hemagglutination by 1 unit of influenza virus (PR8) at 4°C(8).

The present study is concerned with the isolation and characterization of a substance present in normal urine which possesses in extraordinary degree the capacity to inhibit hemagglutination by influenza, mumps and Newcastle disease viruses. Not only is the substance present in the urine of human beings, but it is found in that of the cat, the rabbit, and the guinea pig as well.

Materials and methods. Urine. Urine from normal men was used in most experiments. It was collected in sterile flasks and stored at 4°C.

RBC. Blood from individual chickens was collected into ACD(15) and stored at 4°C. RBC were washed 3 times with 0.85% NaCl buffered at pH 7.2 with 0.01 M phosphate, and diluted to a 1% suspension.

Viruses. FM1, Lee, PR8, swine or NDV was inoculated in 10-day chick embryos. Allantoic fluid was harvested 48 hours later, centrifuged at 3,000 r.p.m. for 20 minutes, the supernate distributed in plastic tubes, frozen, and stored at –70°C. For each test a tube was thawed and the contents used but once. Mumps virus was inoculated in 7-day chick embryos and allantoic fluid was harvested 5 days later.

Hemagglutination tests. Serial 2-fold dilutions of infected allantoic fluid were made in 0.4 cc of buffered saline and 0.4 cc of RBC suspension was added to each tube (final concentration = 0.5%). Tests were read after 1 hour. The last tube showing strong agglutination (2-3+) was considered as the endpoint.

Hemagglutination-inhibition tests. Serial 2-fold dilutions of urine or inhibitor preparation were made in 0.2 cc of buffered saline. Allantoic fluid, diluted so as to yield 2 units of virus in the *final* mixture, was added in 0.2 cc amount to each tube. The mixtures were kept at room temperature for 1 hour, then 0.4 cc of the RBC suspension was added and the tests were read after another hour. The tube showing complete inhibition of hemagglutination was taken as the endpoint.

Experimental. Results of a representative experiment on the hemagglutination-inhibition titers of urine determined with different viruses, both unheated and heated, are shown in Table I. Of the viruses tested, swine influenza was most susceptible to inhibition. PR8, FM1, and Lee gave low inhibition titers before and 64-fold higher titers after heating. NDV yielded the lowest titer of all. With mumps virus 20 minutes of heat-

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TABLE II.
Effects of Treatment of Urine on Hemagglutination-Inhibition Titer.

Treatment of urine*	Inhibition titer†
None	128
Dialysis vs. 0.14 M NaCl	128
" " dist. H_2O	2048
" " " " followed by paper filtration	2048
Paper filtration, undialyzed urine	128
" " , followed by dialysis vs. dist. H_2O	128
Undialyzed urine, 7000 g, 30′, supernate	128
" " " " ", sediment in 0.14 M NaCl	0
" " " " ", " " dist. H_2O	2048
" " 56°C, 30′ (after adjustment of pH to 6.5)	128
" " 70°C, 30′ (after adjustment of pH to 6.5)	2048

* Specimens of urine from 1 normal adult male were used.
† Determined with Lee virus, heated at 56°C, 30′; final concentration, 2 units.

ing at 46°C (heat tolerance of hemagglutination) did not increase susceptibility to inhibition. In addition, urine inhibited hemagglutination with GDVII virus(16) in moderate titer but did not affect hemagglutination with PVM.

Inhibitor titers with RBC from different chickens. Anderson(17) showed that RBC from certain chickens were unsuitable for demonstrating the inhibitory activity of normal serum. A similar phenomenon had been encountered previously(18). RBC from 11 individual chickens were used in inhibitor titrations with a single urine specimen. Both heated Lee and PR8 were employed. With the RBC from 9 chickens, all inhibitor titers fell between 128 and 1024. RBC from 2 chickens showed no capacity to act as indicators for the inhibitor against Lee, although with PR8 usual inhibitor titers were obtained. These observations were confirmed on repeated testing. In all experiments reported in this paper, RBC from individual chickens known to be satisfactory in inhibitor titrations were employed. Guinea pig and human erythrocytes also can be used satisfactorily in inhibitor experiments with urine.

Effects of treatment of urine. In most instances the actual concentration of inhibitor is higher than is indicated by the titer of the untreated urine. Table II shows that only about 6% of the inhibitor could be accounted for when fresh untreated urine was employed. The titer after dialysis against water or after heating at 70°C when the pH was adjusted to 6.5 was 16 times higher than prior to such treatment. However, the titer did not increase after dialysis against 0.14 M NaCl, or when dialysis was preceded by filtration through paper. Centrifugation of untreated urine at 7,000 g caused no reduction in the inhibitor titer, but yielded an appreciable amount of mucoid sediment which gave no inhibition when resuspended in saline. When the sediment was resuspended in distilled water, it showed a high inhibitor titer. Urine of low specific gravity (*e.g.* 1.005) showed no rise in inhibition titer on dialysis against water. On the average such urine showed a titer of 512. Urine with high specific gravity (*e.g.* 1.028) showed marked increase in titer on dialysis, *e.g.*, from 16 to 8192. Thus, specific gravity can be used as a criterion for predicting the change in inhibitor titer which occurs on dialysis.

Comparison of inhibition titers of heated (70°C) or dialyzed morning urine from 7 normal men against Lee and PR8 revealed that the titers did not differ markedly, ranging from 1024 to 8192. The differences were correlated directly with variation in specific gravity. Inhibition titers of dialyzed urine obtained on different days from the same persons showed similar variation. Urine

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from normal women gave inhibition titers comparable to those from men. In addition, urine from cats showed inhibition titers of moderate degree; that from rabbits and guinea pigs gave lower titers. When sediments obtained by centrifugation of fresh urine were dissolved in distilled H_2O, they gave high inhibitor titers. However, when to such a solution NaCl was added to 0.58 M and the mixture held at 4°C for 24 hours, no free inhibitor could be detected. Under these circumstances the solution became turbid; if the inhibitor concentration was sufficiently high, precipitation of the inhibitor occurred. As the amount of salt added was reduced so as to yield 0.29 M or less, increasing amounts of free inhibitor were demonstrable and the titer at 0.036 M NaCl was equal to the maximum titer of the urine after dialysis against water. The progressive decrease in inhibitor titer in the presence of salt concentrations of 0.14 M or more is a slow process and may not reach equilibrium for long periods (1-2 days); the higher the salt concentration, the shorter the period required. However, the increase in inhibitor titer at low salt concentrations is very rapid and occurs in a few minutes.

Quantitative relationship between inhibitor and virus. When the quantities of urine and heated Lee virus employed in inhibition experiments were varied with respect to each other, the results illustrated in Fig. 1 were obtained. The relationship between the two variables was one of multiple proportions only when both were in high concentration. At lower concentrations the ratio between virus and inhibitor became 2:1 and at extreme dilution may be as great as 3:1. This phenomenon was not due to loss of hemagglutinating activity of virus at high dilution because there was no appreciable drop in virus titer when comparable dilutions of allantoic fluid were kept at room temperature for 10 hours. Similar experiments were carried out with a urine dialyzed against water which caused a 32-fold increase in inhibitor titer. The results were closely similar to those shown in Fig. 1. When mixtures of varying quantities of inhibitor and virus were held for 12 hours before the addition of RBC, the relationship between the two variables had the same form as shown in Fig. 1. However, the inhibitor titers were increased 4-fold. This is in agreement with the progressive rise in inhibition titer with time described below.

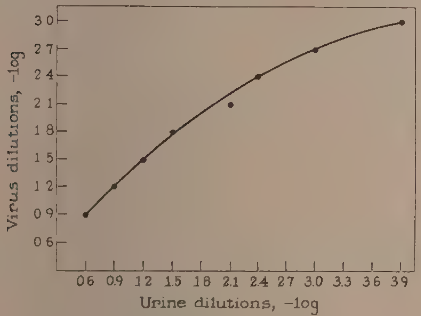

FIG. 1.

Quantitative relationship between urine inhibitor and virus. Heated (56°C, 30′) Lee virus, from 1 to 128 units, and dilutions of normal untreated urine were employed. The urine showed no increase in inhibitor titer on dialysis against distilled H_2O.

Increase in inhibition titer with time. Mixtures of urine dilutions and a constant amount of either Lee or PR8 (4 units per tube) were kept at room temperature for periods up to 21 hours before addition of RBC. Control titrations were carried out simultaneously by mixing inhibitor and virus, each of which had been kept separately in comparable dilutions under identical conditions. In addition, control virus titrations were carried out with comparable dilutions which were held for equally long periods. Results of a typical experiment with Lee virus are shown in Fig. 2. The inhibitor titer showed a rapid rise during the course of the first hour. During the following 11 hours the rise continued, but at a much slower rate. Maximal inhibitor titers were not reached until 12 hours. Control titrations with virus and inhibitor dilutions kept separately for similar periods showed no increase in titer when mixed, nor did the hemagglutination titer of the virus suspension decrease during the course of the experiment. Identical results were obtained with a dialyzed urine that showed a 16-fold rise in titer after dialysis, as well as with a purified prepara-

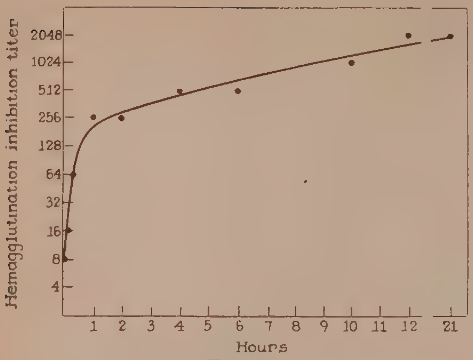

FIG. 2.

Increase in inhibitor titer with increase in time of interaction between inhibitor and virus. Heated (56°C, 30′) Lee virus, 4 units, and dilutions of normal untreated urine were employed. The urine showed no increase in inhibitor titer on dialysis against distilled H_2O.

tion of inhibitor. Moreover, dialyzed urine and the purified preparation, when tested at 4°C, gave closely similar results. Thus, titrations of the inhibitor should be allowed to react for 12 hours before addition of RBC if the system is to reach equilibrium. For practical reasons, a 1-hour period of interaction was employed in this study. Lanni and Beard(6) demonstrated that with egg-white and swine influenza virus maximal titers are obtained after about 30 minutes of incubation at 28°C. Subsequently, they showed(14) that the inhibitor titer of cow's milk increased as incubation was prolonged to 90 minutes.

Pseudo-hemagglutination. When RBC from some chickens are mixed with certain urine specimens, agglutination of RBC occurs in the absence of virus. Usually the titers are low(2-8) but occasionally values of 16 to 32 are obtained. Such pseudo-hemagglutination can interfere with the determination of the inhibitor titer, particularly if the latter is low. The pattern of pseudo-hemagglutination is variable: it can closely resemble that caused by viruses, but more frequently it is coarser in type. Heating urine at 70°C for 30 minutes eliminates pseudo-hemagglutination, regardless of whether the RBC or the urine was the factor of greater importance.

Destruction of inhibitor. Lee or PR8 virus as well as cholera vibrio filtrate destroys the inhibitory activity of urine upon incubation at 37°C for 1-2 hours. Pneumococcal hyaluronidase in a concentration of 0.001 mg/cc eliminates inhibitor activity under similar conditions. Crystalline trypsin in a concentration of 0.5 mg/cc had no effect on inhibitor titer after incubation at 37°C for 24 hours; after 7 days, however, the titer was reduced by 99%. Lithium periodate in a concentration of 0.00025 M reduced the inhibitor titer by 98% in 2 hours at 23°C. Heating dialyzed buffered urine at 70°C for 8 hours caused no drop in inhibition titer. Heating at 80°C for 30 minutes reduced the titer by 98% but after 2 hours there was no further reduction. Similar results were obtained at 100°C; after a rapid drop in titer (marked reduction was demonstrable at 1 minute), no further reduction took place during 2 hours.

*Purification of inhibitor.** *Precipitation with alcohol.* The addition of 6 volumes of 95% ethyl alcohol gave the best yields of inhibitor. The mixtures were held at 22-24°C and the precipitates obtained were redissolved in water. To obtain maximal yields, it was necessary either to use undialyzed urine or to employ 9 times concentrated urine which was dialyzed before precipitation. The results of one experiment were: To 2 liters of urine was added 12 liters of 95% alcohol and the mixture held at 22-24°C for 48 hours. The precipitate was collected in the centrifuge, and dissolved in 200 cc of distilled H_2O. A small amount of inert material was separated by centrifugation and the supernatant removed, concentrated to 40 cc *in vacuo* and dialyzed against distilled H_2O at 4°C. The material (P) was dried from the frozen state: 115 mg of a pale brown powder, readily soluble in H_2O was obtained. The inhibition titer at a concentration of 1 mg/cc was 128,000 against 2 units of heated Lee. Analytical tests yielded: Molisch reaction: negative before but positive after acid hydrolysis; biuret test: negative; nitrogen: 9.8%; phosphorus: 0.94%; ultraviolet absorption curve: peak at 2750 Å.

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* Dr. Walther F. Goebel, The Rockefeller Institute, kindly cooperated in the purification studies.

Fraction P was further purified as follows: a 0.75% solution, containing 85 mg and 5% of sodium acetate, was shaken 5 times with chloroform-octyl alcohol mixture (4:1)(19). The supernatants (S) were collected and pooled. The cakes in the chloroform layer also were combined, mixed with an equal volume of 95% ethyl alcohol, and the precipitate (C) was collected by centrifugation and redissolved in water. This and the pooled supernatants (S) were dialyzed against water. The yields were: S = 23 mg; C = 34 mg. The inhibitor titers at concentrations of 1 mg/cc were 128 and 4,096,000, respectively. Comparing these values with the titer of fraction P: *i.e.* 128,000, it appears that only 0.1% of activity remained in the aqueous phase (S). On the other hand, the precipitate (C), which represented less than half of fraction P on a weight basis, showed an inhibitor activity 32 times greater than that of P.

The analytical data with the highly active fraction (C) were: Molisch reaction: positive without acid hydrolysis; biuret test: positive; nitrogen: 12.5%; phosphorus: 0.00%. Ultraviolet absorption curve showed a peak of the same height as given by fraction P with a maximum at 2800 Å, and considerably accentuated minima on both sides. The relatively inactive aqueous phase fraction (S) was biuret negative, but Molisch positive and the ultraviolet absorption curve showed no peak at 2800 Å. Computations showed that 1 unit of heated Lee was inhibited by 0.004 μg of the original alcohol precipitate (P); by 0.4 μg of the material that remained in the aqueous phase (S); and by 0.0001 μg of the substance obtained from the chloroform phase (C).

Isoelectric precipitation at pH 3. A 2.5% solution in water of material obtained by alcohol precipitation gave a precipitate when the pH was lowered to 3 with 1 N HCl. After reprecipitation, the material was dissolved in water, brought to pH 7.0 with 1 N NaOH and frozen and dried *in vacuo*. This acid insoluble fraction represented half of the starting material by weight, but was twice as active in inhibition tests. When undialyzed urine was brought to pH 3, only a small amount of precipitate was obtained which showed low activity. However, when 2 liters of urine was concentrated 40 times, and then precipitated in the same way, 60 mg of a slightly brownish material was obtained which showed an inhibitor titer of 256,000 (1 mg/cc; 2 units heated Lee). Comparing this result with that obtained when 2 liters of urine was precipitated with alcohol, it appears that acid precipitation yields material twice as active as and less pigmented than the alcohol procedure (fraction P).

Sodium chloride precipitation. As stated above, the inhibitor in urine is precipitated in the presence of 0.58 M NaCl. This property was used in separation of the inhibitor by filtration and centrifugation. Filtration through porcelain candles or fritted glass discs in the presence of sufficient salt provided separation of inhibitor and urinary pigment. When a urine (specific gravity 1.010) was filtered through a Coors No. 3 candle, no inhibitor appeared in the filtrate. The filter candle was then washed with 0.14 M NaCl until no pigment appeared in the washings. Such washings contained no inhibitor. When distilled water was passed through the filter, all the inhibitor was released. The final water wash (equal in volume to the urine filtered) showed an inhibitor titer 64 times higher than the untreated urine. Similar results were obtained with Selas No. 2 filter candles and with fritted glass filters (Corning). With large volumes of urine *medium* fritted glass filters gave satisfactory results and high yields of inhibitor. Best results were obtained when urine was diluted with 1 volume of distilled H_2O, NaCl was added in a concentration of 0.58 M, the pH was 5-6, and the urine was held at 4°C for 24 hours prior to filtration. The substance is highly viscous and centrifugation in an angle head may cause part of the material to adhere along the wall of the tube. However, the bulk of inhibitor in urine (specific gravity 1.020) was recovered in the sedi-

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ment after centrifugation at 7,000 g for 30 minutes. After washing with 0.58 M NaCl and recentrifugation, the sediments were frozen and dried. In two experiments a titer of 64,000 (1 mg/cc; 2 units heated Lee) was found.

Discussion. The presence in normal human urine of a potent inhibitor of viral hemagglutination makes readily available a rich source of this substance and one essentially free of protein. Separation of the inhibitor from urine can be accomplished without difficulty and yields of purified material, active at 0.0001 μg/cc, are of the order of 17 mg/liter.

The chemical properties of this substance indicate that it has much in common with similar inhibitors found previously in other biological materials and suggests that, like these, it too is a mucoprotein. The striking effects of salt concentration upon the activity of the inhibitor as well as upon its filterability and sedimentability appear to be referable to its solubility properties in electrolyte solutions. This view is supported by the finding that the inhibitor is precipitated in NaCl solutions of 0.6 M. However, there is also evidence to suggest that a heat labile component in urine may combine with the inhibitor in the presence of salt and lead to some of the observed effects.

Summary. Normal human urine contains a highly active inhibitor of viral hemagglutination, effective against PR8, Lee, FM1, swine, mumps, NDV, and GDVII viruses but inactive against PVM. The inhibitor concentration may approach 2 mg%. Considerable amounts of purified material, active at 0.0001 μg/cc, can be obtained readily. The activity of the inhibitor as it occurs in urine is inversely related to the salt concentration; it should be emphasized that in NaCl solutions of moderate strength it precipitates.

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A Feathering Syndrome in Chicks After Feeding Optimal Levels of Lysine in Absence of Arginine. (17826)

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It has been shown that arginine is important for the prevention of an abnormal feathering syndrome caused by a glycine deficiency in chicks(1) and that a lysine deficiency causes an abnormal pigmentation of the feathers in poults(2). During nutritional studies with milo gluten meal (a 44% protein byproduct of starch manufacture from milo and other grain sorghums) a peculiar feathering abnormality developed in chicks fed a diet containing 50% milo gluten meal supplemented with 2% DL-lysine. Milo gluten meal was the only source of protein in the above diet. This syndrome developed consistently in 5 successive groups of experimental chicks. This study was initiated to determine the nature of the abnormal feathering syndrome.

Experimental. New Hampshire chicks were used in these experiments. The chicks were housed in electrically heated battery brooders with raised screen floors. Feed and water were supplied *ad libitum.* The chicks were wingbanded when one day old, observed daily, and weighed weekly for a period of 4 weeks.

The basal diet (MG-1) contained 50% milo gluten meal (calculated to provide 22% crude protein), 4% salts IV, 3% corn oil,

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* Supported by a grant-in-aid from Corn Products Refining Company, Argo, Ill.

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TABLE I.
A Comparison of the Amino Acid Content of the Basal Diet (MG-1) with the Amino Acid Requirement of the Chick.

	Biologically active amino acids in basal ration, %	Amino acid requirement of chick (% of diet)*
Arginine	0.48	1.2
Histidine	0.35	0.15-0.30
Isoleucine	1.02	0.6
Leucine	3.94	1.4
Lysine	0.32	0.9
Methionine	0.37	0.9
Phenylalanine	1.25	0.9
Threonine	1.22	0.6
Valine	1.20	0.8
Tryptophane	0.18	0.18-0.25

* Almquist, H. J., *J. Nutrition,* 1947, v34, 543.

2% fish oil (3000A-400D), and 41% corn starch. The following vitamins were added in mg per kg of diet: pyridoxine—3.0; biotin—0.2; thiamin—4.0; riboflavin—6.0; calcium pantothenate—15.0; niacin—100.00; alpha tocopherol—3.0; folic acid—2.0; choline chloride — 2000.0; menadione — 0.5; Merck APF No. 3 (calculated B_{12} 27.5 μg/kg)—1000.

Six groups of 15 chicks each were used in each test. Two such experiments are reported herein. The first group was fed diet MG-1. Four other groups were fed diets MG-9 (2% DL-lysine), MG-17 (2% DL-lysine, 0.54% DL-methionine and 1% glycine), MG-18 (2% DL-lysine, 0.54% DL-methionine, 1% glycine and 0.6% L-arginine) and MG-19 (2% DL-lysine and 0.6% L-arginine) respectively while the sixth group received diet MG-4 (18% crude casein and 10% gelatin).

The amino acid content of the milo gluten meal was determined by standard microbiological procedure(3).

Results and discussion. The amino acid content of diet MG-1 is shown in Table I. The percentages of amino acids in this instance were calculated from the microbiological assay data for the 10 essential amino acids listed. These values are given for the purpose of comparison with the amino acid requirements of the chick, according to Almquist(4). It is immediately apparent that the basal diet (MG-1) is deficient in arginine, lysine, and methionine.

Structural feathering abnormalities were not noted in any case where diet MG-1 was fed. There was a slight degree of depigmentation in the feathers of the chicks fed diet MG-1 and this was probably due to the lysine deficiency of this diet since it has been reported that a deficiency of lysine will cause a depigmentation in the feathering of bronze turkeys. It appears probable that the feathering abnormalities observed in this study when the basal diet was supplemented with lysine (diet MG-9) or with lysine, methionine, and glycine (MG-17) might have been due to the deficiency of arginine and that the addition of lysine precipitated an arginine imbalance or deficiency, which resulted in the abnormal feathering observed. Such an assumption is substantiated by the fact that arginine added with either of the above combinations (MG-18 and 19) corrected the feathering abnormalities. The work in this study was concerned only with New Hampshire chicks. However, the feathering abnormalities described have also been observed in crossbred chicks (NH males x SCWL females). When diet MG-9 (DL-lysine) was fed, the feathering of the chicks was decidedly abnormal in appearance (Fig. 1 and 2). A detailed description of the abnormal conditions of the feathers in these birds appears below. Supplementation of the basal diet with lysine, methionine, and glycine (diet MG-17) likewise resulted in the typically abnormal feathering associated with the inclusion of lysine alone in the basal diet. When the basal diet was supplemented with 2% lysine and 0.6% arginine, feathering of the birds was normal in all instances. Supplementation of the basal diet with lysine, arginine, methionine, and glycine (MG-18) likewise resulted in normal feathering. In addition it should be pointed out that the feathering of birds fed diet MG-4 (18% casein and 10% gelatin) was normal in all respects. Growth studies of the birds

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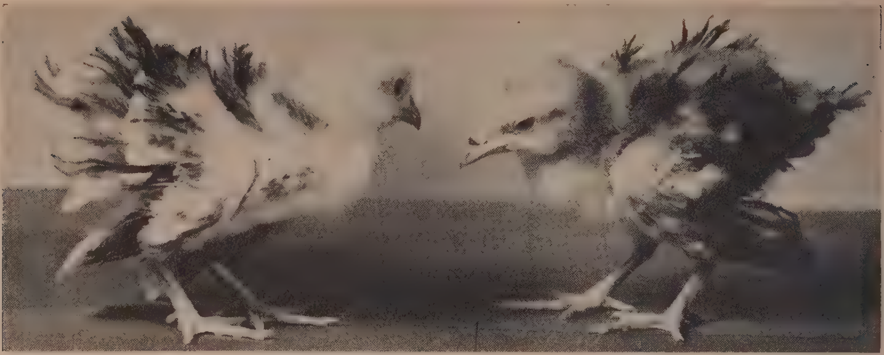

FIG. 1.
Two chicks fed diet MG-9 (2% DL-lysine). Note ruffled feathers.

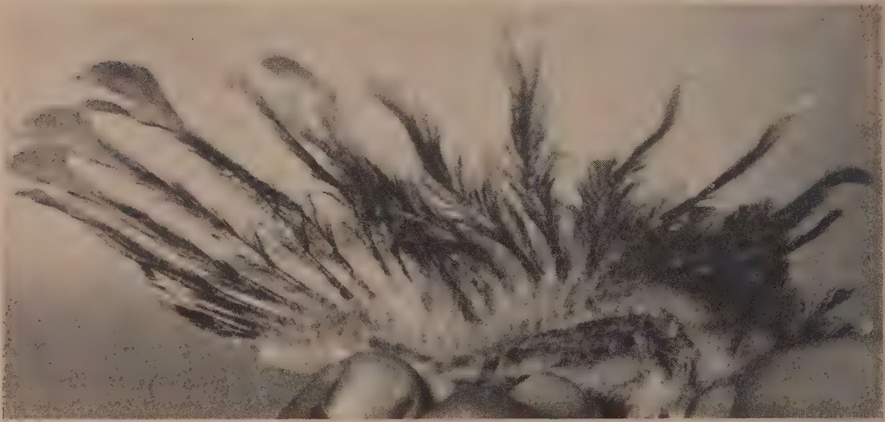

FIG. 2.
Wing feathers of a chick fed diet MG-9 (2% DL-lysine).

in these tests and others, in which milo gluten meal was used, will be reported elsewhere.

The chicks fed diet MG-9 and MG-17 exhibited an extremely abnormal feathering at 4 weeks (Fig. 1 and 2). All of the feathers were spoon-like in appearance. This was apparently caused by the retention of an abnormally long feather sheath which covered the proximal third or more of the feather shaft. The spoon-like appearance was accentuated by limp and degenerate barbs and barbules along the proximal 4/5 of the feather not covered by the abnormally long sheath. The integrity of the vane was maintained only at the tip by normal barbs and barbules. The abnormally small and flexible rachis followed the normal feather contour for about one-half the length of the feather and then was bent sharply outward. The primary wing feathers were turned so that the arc described by the rachis pointed downward and the distal ends of the primaries almost dragged on the bottom of the cages. The secondary wing feathers were turned in the opposite direction and the feather tips were placed well above the level of the back.

Attention is called to the fact that DL-lysine only was fed in these tests. The question as to whether the abnormal feathering reported herein would have occurred with the feeding of D- or L-lysine in a diet low in arginine remains unanswered.

Summary. The addition of lysine to a chick diet deficient in lysine, arginine, and methionine caused an abnormal feathering which was characterized by curved and degenerate feathers. A detailed description of the feathering abnormalities is given. When arginine was added, along with the lysine, the feathering of the birds was normal. The addition of the lysine without arginine apparently created a severe deficiency or imbalance of the latter amino acid which resulted in the abnormal feathering.

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Determination of Inulin by Means of Resorcinol. (17827)

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Despite numerous difficulties encountered in its use, inulin has remained the substance of choice for the determination of the glomerular filtration rate in man. The chief disadvantage has been the necessity of yeasting plasma and urine in the diphenylamine method(1), a step which is both cumbersome and a potential source of variable errors. Any method that avoids yeasting would be advantageous to renal physiologists and clinicians. Several investigators have examined the applicability of the reaction of resorcinol with fructose(2-4) to the determination of inulin(5-8) but differences in technic imply that methodological errors have not been reduced to a minimum. Accordingly it was decided to investigate the resorcinol method further and to compare it with the diphenylamine method under clinical conditions. Since this work was initiated, Roe(9) has reported an adaptation of the resorcinol method and we have included a comparison of our final method with this later procedure. Since completion of this work, Rolf *et al.*(10) have presented a modified diphenylamine procedure for fructose and inulin determinations. Their method advantageously eliminates the yeasting step but maintains the disadvantages of the tedious purification of diphenylamine, long heating period, and danger of crystallization of diphenylamine after cooling. Moreover, the method herein described yields a higher inulin/glucose color ratio and consequently lower blank values than the method of Rolf *et al.*

Conditions for the determination of inulin. Since the determination of inulin by the resorcinol reaction depends upon the hydrolysis of inulin to fructose, the strength of acid employed in the hydrolysis and the time and temperature of heating will all be critically important.

As judged by color development in a standard procedure using known amounts of inulin, we have found that 15 to 18% hydrochloric acid in the final reaction mixture gives maximal color development. It would appear that at this concentration maximal hydrolysis is achieved, and at the same time optimal conditions are set up for the reaction of fructose with resorcinol(3). As the concentration of acid is reduced color development becomes progressively less, while at higher concentrations slightly increased color

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development is attained but the effect of interfering substances in plasma becomes proportionately greater at concentrations of HCl above 18 per cent in the final mixture. Using a final concentration of 17% acid, maximum color development occurs when the hydrolysis is carried out at a water bath temperature of 80°C. Below this temperature color development is progressively less until at 40°C and below no color is developed, while at 100°C the reaction produces the pale yellow color described by Kruhoffer, instead of the red color characteristic of the reaction at lower temperatures. Color development increases as heating is prolonged from 5 to 20 minutes in a water bath at 80°C, but extending the period to 25 minutes does not alter the intensity, and heating for as long as 35 minutes gives color only slightly less intense than at 20 to 25 minutes. In our hands, therefore, the optimal conditions for the development of the red color comprise 17% hydrochloric acid in the reaction mixture, and heating for 25 minutes in a water bath at 80°C. These conditions differ from those of Roe's earlier method in that we heat longer, use slightly less acid and read in a spectrophotometer as described.

Although the red color follows the Beer-Lambert law rather closely in concentrations up to 2 mg/100 cc, the slope of the curve decreases somewhat variably with greater concentrations. We have therefore followed the usual practice of including with each batch of unknowns 3 standards in duplicate (1, 2 and 3 mg/100 cc) and establishing a standard curve for each set of determinations.

There are several locations on the absorption spectrum of this color which may be used for a spectrophotometric reading, a circumstance reflected in the fact that each of the previous investigators has chosen a different wave-length. There are two peaks on the absorption curve, one occurring at 410 μ and another 490 μ. We have chosen 490 μ as the reading point.

Methods. Based upon the above results we suggest the following procedure: A 2 cc aliquot of standard or unknown is pipetted into a 15 x 200 mm Pyrex ignition tube. Two cc of a 0.1% solution of resorcinol in 95% alcohol are added, this reagent being prepared daily. Finally, 5 cc of 30% hydrochloric acid are added. (This solution is prepared by adding 22.4 cc of water to 100 cc of concentrated acid.) The mixture is shaken and a glass tear placed in the mouth of the tube. The tubes are placed for 25 minutes in a water bath at 80°C,* after which they are cooled in tap water for 3 minutes and closed with a rubber stopper. Color intensity is read in a Coleman Junior Spectrophotometer at a wave length of 490 μ with an aqueous reagent blank set at 100% transmission. The color developed in this fashion is reasonably stable for periods up to 85 minutes, but routinely they should be read promptly. It is best to pour the reaction mixture in the cuvette at a distance from the colorimeter, and to keep the cuvettes stoppered, because the strong acid fumes may attack the colorimeter.

Two cc of standard solutions containing 1, 2 and 3 mg/100 cc of fructose-free inulin are treated in an identical manner for the determination of the standard curve.

Determination of inulin in plasma. In the precipitation of plasma, 2 cc of plasma are placed in a 250 cc narrow mouth Erlenmeyer flask, water for the appropriate precipitation dilution (for a given plasma inulin level) is added and mixed, followed by 6 cc of cadmium sulfate solution† with agitation and then 2 cc of 1.1 *N* NaOH solution which is added dropwise, again with agitation. The flasks are then stoppered, shaken well and allowed to stand for 10 minutes with some additional shaking. The precipitate is separated by centrifugation and the supernatant fluid is filtered through washed cotton.

Aqueous recoveries. Solutions of inulin containing 15, 30 and 45 mg/100 cc respectively were treated as described for plasma, to determine if the cadmium sulfate filtrate is suitable for use. Analysis of these aqueous filtrates was carried out as above. In 7 experiments at each inulin concentration, the

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* The water bath we employed had a temperature variation of ±1°C.

† 34.67 g $3CdSO_4 \cdot 8H_2O$ plus 169 cc 1.0 N H_2SO_4 (exact) are made up to 1000 cc and filtered if necessary.

TABLE I.
Comparison of Inulin Clearances by Diphenylamine and Resorcinol Methods.*

Conditions	No. of determinations	Avg ratio†	Standard deviation‡
During PAH clearance	30	1.006	.0822
During PAH Tm determination	16	1.004	.0942
During glucose Tm determination§	3	1.067	insufficient determinations

* Expressed as inulin clearance determined by the diphenylamine method(1) divided by the inulin clearance as determined in the same plasma and urine samples by the resorcinol method.

† Mean of the series.

‡ Square root of the average of the squares of the individual deviations.

§ Uncorrected for either plasma or urine glucose levels.

average aqueous recovery was 98% (range 94 to 101), with no consistent deviation from this figure at any particular concentration. To determine the plasma inulinoid blank blood samples were taken from 24 normal subjects. The plasma was precipitated 1 to 10 as described above. The inulinoid blank averaged 1.59 mg/100 cc (range 1.05 to 2.70) in inulin equivalent. Nine subjects in this group were undergoing alimentary glucose tolerance tests and were known to be in a post-absorptive state. In these the blank averaged 1.33 mg/100 cc. In those subjects undergoing an Exton-Rose glucose tolerance test, the inulinoid blank increased in proportion to the hyperglycemia. In 5 subjects who had ingested a high carbohydrate meal the blank increased far out of proportion to the hyperglycemia, reaching in one subject a value equivalent to 6.3 mg/100 cc. We interpret this to be due to a larger fraction of circulating fructose following the high sucrose meal. Since these data indicate that in a fasting state, most of the plasma inulinoid blank can be attributable to glucose, standard solutions of the latter containing 50, 100, 120, 150 and 200 mg/100 cc of glucose were analyzed. These solutions developed color equivalent to 0.55, 1.18, 1.34, 1.70 and 2.25 mg/100 cc of inulin. In one subject studied hourly from early morning to late evening, the blank varied from 0.75 to 1.90 mg/100 cc, the high value being reached after the evening meal.‡ Recovery of added inulin from plasma was examined by adding standard solutions of inulin to the plasma-water mixture during precipitation. The quantities of inulin so added corresponded to plasma inulin concentrations of 15, 30 and 45 mg/100 cc. The plasma inulinoid blank was determined directly on a separate sample. In 7 experiments at each concentration the average plasma recovery for all concentrations was 99% (range 95 to 104), with no consistent deviation from this average at any one concentration.

The resorcinol method was compared with the diphenylamine method under clinical conditions, using blood and urine samples from patients whose renal function was being routinely evaluated. Inulin was determined by the resorcinol method in our laboratory, while determinations by the diphenylamine method were carried out in an independent laboratory routinely employing this method. The results of the comparison are shown in Table I. Because the diphenylamine determinations were performed on a different spectrophotometer against a different standard curve the clearance values rather than the absolute plasma values are used as a basis for comparison.

Simultaneous determinations were also compared in 2 non-fasting subjects undergoing 24 hour clearance tests. The plasma ratios did not give satisfactory values, presumably because of the diurnal fluctuations in the plasma inulinoid blank. The ratio of the urinary inulin concentration as determined by the two methods in 12 periods was 1.005.

In his later method Roe(9) recommended a resorcinol reagent having glacial acetic acid as the solvent and containing thiourea as well as resorcinol. Using a Somogyi filtrate, Roe reported recoveries of inulin from plasma ranging from 99 to 102%. Since many clinical investigations would be facilitated by the simultaneous determination of inulin and p-aminohippuric acid, we have examined the applicability of the Roe method to the cadmium sulfate filtrates routinely used in the determination of p-aminohippuric acid. Here plasma recoveries averaged 101% and aqueous recoveries averaged 99% when carried out at concentrations simulating 15, 30 and 45 mg/100 cc of inulin in plasma. Using 3 aqueous and 3 plasma filtrates and making 4 determinations by each method on each filtrate, the Roe method gave an average inulin recovery identical with the average recovery as determined by our method. The maximum discrepancy on any filtrate was 2.8%, while the average of 4 recoveries was 101.3% by the Roe method and 98.5% by our method.

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‡ In 3 post-prandial subjects the clearance of plasma inulinoid blank ranged from 1.89 to 3.42 cc per minute. Thus although plasma inulin values must be corrected for an inulinoid blank, the urine correction in normal subjects is relatively insignificant.

The plasma inulinoid blank as determined by the Roe method appears to be slightly higher than that determined in our method. For example, the average inulinoid blank on 5 post-absorptive plasma samples was equivalent to 1.42 mg/100 cc inulin by the Roe method and 1.34 mg/100 cc of inulin by our method, and in no sample was the blank less by the Roe method. Roe's glacial acetic acid-urea method appears from these limited data to be equally as good as our modification of the resorcinol method. Our method has the advantage of somewhat greater simplicity.

Discussion. The resorcinol method described above, and the later method of Roe, offer several advantages over the diphenylamine method for the determination of inulin. They consume less time and are less tedious because yeasting is avoided. Resorcinol can be obtained commercially in a pure form, whereas diphenylamine must almost invariably be recrystallized. The fact that cadmium sulfate filtrates of plasma can be used to determine plasma inulin concentration is a distinct advantage where either the PAH clearance or Tm_{PAH} is determined simultaneously with inulin.

The plasma inulinoid blank in the post-absorptive state has the same order of magnitude in the diphenylamine and resorcinol methods. However, after the ingestion of food the blank may fluctuate in the resorcinol method because of the contribution of fructose. To reduce the significance of fluctuations under this circumstance it is recommended that high plasma levels of inulin (20 to 30 mg/100 cc) be employed.

With the urine dilutions used in routine clearance studies no correction is necessary for urinary chromogen. However, undiluted urine shows a significant obsorption at 490 μ and this absorption is intensified when HCl is added, even in the absence of resorcinol. In the study of undiluted urines this chromogen may be removed(3) or a correction made by reading urine samples against a urine pigment blank composed of urine and acid without resorcinol.

In using any inulin method which does not involve the use of yeast to remove fructose, it is imperative that fructose-free inulin be used for renal clearance tests.

Summary. A simplified method, based on Roe's resorcinol method and avoiding the use of yeast, is described for the determination of inulin in plasma and urine. The method has been applied under clinical conditions to clearance determinations in patients with renal disease, and shown to yield results in good agreement with the diphenylamine method.

We are pleased to acknowledge the assistance of Mr. Jerome Schulman and the technical aid of Joanne Baker Schreiner and Karin Delmonte; we are indebted to Dr. David Earle Jr. and his colleagues, Dr. J. Deaver Alexander and Dr. Saul Farber who supplied the samples and performed the yeast-diphenylamine determinations on which our comparisons are based.

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Acid-Fast Membranes of Lipid Pneumonia by Fluorescence Microscopy. (17828)

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An insoluble membrane may be formed around certain oil globules within a protein solution or lung tissue, that stains with the acid-fast technic. Such membranes have been described for unsaturated oils like cod liver, paraffin oil and rabbit fat in the lung and in serum(1,2,3). The increased sensitivity of the auramin fluorescence for revealing acid-fast bacteria suggested that it should be useful in the examination of tissues from cases of lipid pneumonia(4) due to aspiration of unsaturated oil, *e.g.* cod liver oil.

Tissue embedded and sectioned in paraffin was mounted, deparaffined and stained in 0.1% Auramin O (C.I. No. 655) in 3% phenol for 2-5 minutes and destained in salt-acid alcohol (0.5% NaCl in 70% EtOH with 1 ml HCl) until the dye remained only in the acid-fast material; usually in 5 to 10 minutes with at least 2 changes of the bleach. On examination with long wavelength ultraviolet radiation the auramin in the acid-fast material fluoresces strongly yellow, Fig. 1B-D, revealing the membranes from the lipid pneumonia rather better than does the usual Ziehl-Neelsen staining, Fig. 1A, with carbolfuchsine. As the eye sees small bright details on a dark background better than the converse, the finer details are readily visible. Thioflavine TG (C.I. No. 815) may also be used. Sections stained in 1942 and mounted with a cover glass held in place at the edges by gold size had not faded by 1950. Polyvinyl alcohol may be used without a cover glass to make a lasting mount, but mounting in balsam, etc. is not satisfactory because the balsam itself fluoresces and spoils the contrast and the auramin gradually fades in cemented mounts(5).

The brightest fluorescence is obtained with the crossed filter method using a blue-ultraviolet transmitting filter on the radiation source or the condenser of the microscope and a complementary yellow filter in or on the eyepiece of the microscope. A mercury arc, or a high amperage tungsten filament lamp, may be used as sources although the latter is suitable only for a monocular microscope(6). The acid-fast material is seen as bright yellow on a yellowish background, since the blue-white fluorescence of the protein in the tissues is seen as yellow. For color differentiation a Corning No. 5860 filter may be used with the Hg arc which transmits no visible light. Other combinations are possible and fluorescing counterstains may also be used(5). The intensity of the fluorescence image is low, despite the high contrast, and best results will be obtained when working in a room with subdued illumination.

Ceroid, an acid-fast, sudanophillic hyaline material formed in certain instances of experimental cirrhosis found in macrophages within the liver(7) should be visible also with this technic. Serum and lipid combinations have been reported to increase antibody formation(8) and may prove to be acid-fast. Meyer(9) has discussed acid-fast materials found in animal tissues, especially those reported by Cuénot(10) in the connective tis-

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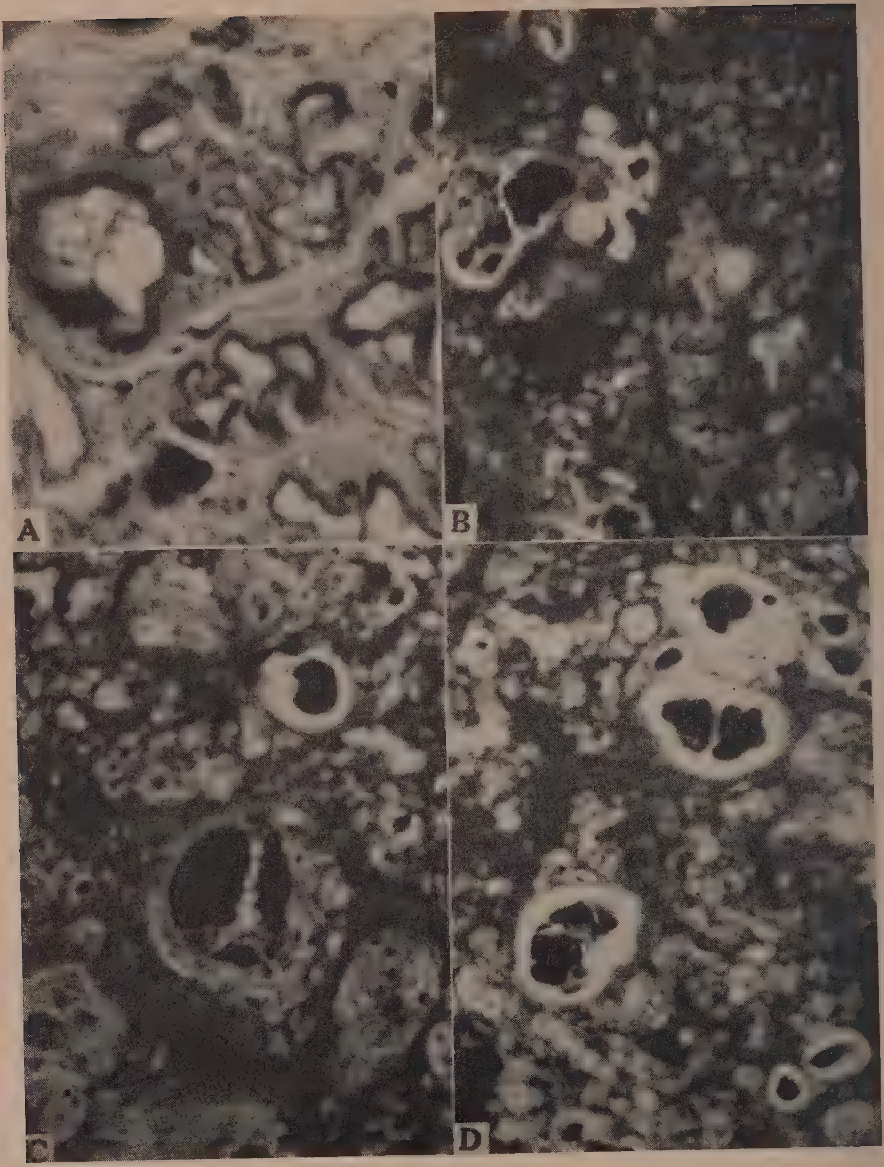

FIG. 1.
Lung sections showing acid-fast material from lipid pneumonia due to cod-liver oil, 300×.
A. Ziehl-Neelsen stained section; B-D, Fluorescence image after treatment with carbol-auramin.

sue of the earthworm with regard to the possibility of their being symbiotic bacteria. The fluorescence microscope should be useful for the examination of these materials and should aid in understanding the phenomena involved.

Summary. Fluorescence microscopy using carbol-auramin or carbol-thioflavine which are specific for acid-fast materials is recommended for the study of the membranes formed in lipid pneumonia where unsaturated lipids are suspected and for other familiar lipo-protein combinations, as more sensitive than the brightfield microscopy with Ziehl-Neelsen staining.

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Some Biochemical Aspects of Herpes Infection.* (17829)

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Inoculation of an adapted strain of herpes simplex virus on the chorio-allantoic membrane of an egg is followed by viral growth not only in this membrane but also in the liver, heart, spleen, and brain of the embryo (1). The pathology and histology of the herpetic infection of these tissues has been described by Anderson(1) while the pathology of the same infection in tissue culture has been given by Rivers, Haagen and Muckenfuss(2). This work is a preliminary study of some of the biochemical changes which characterize the host tissue during the course of this viral infection. It was from the viewpoint of learning the nature of the alterations which take place in the metabolism of the infected tissues that measurements were made: of the rate of growth of the tissues; of two of the respiratory enzyme systems; of changes in the nucleic acid composition; and of the protein content. The results of these findings and some of their significance are discussed in the following.

Experimental. In all experiments the Armstrong 1166 strain of herpes simplex virus was used. Previously it had undergone 54 passages in mice and 37 passages in eggs. The inoculation was made on the chorio-allantoic membrane of 12-day-old embryonated eggs. The tissues were harvested after 3 days and enzyme determinations were made on pooled samples of this moist tissue. Other pooled samples were frozen and brought to dryness *in vacuo*. These desiccated samples were used for nucleic acid and nitrogen determinations.

Methods. The protein nitrogen was determined on samples of tissue which were precipitated from an aqueous suspension with trichloroacetic acid and washed repeatedly with alcohol and ether. The nitrogen was measured by the Koch-McMeeken modification of Nessler's method(3). To determine the number of nuclei per unit weight of liver, a 10% homogenate of the tissue was prepared in 1.0% citric acid(4). The citric acid hardened nuclei were stained with methyl green and counted in a hemocytometer after appropriate dilution. The phosphate was determined by the modified Fiske and Subbarow procedure(5). Succinoxidase determinations were made using the method of Potter and Schneider(6). The α-ketoglutaric acid oxidase activity was measured by the method described by Ackermann(7). The desoxyribosenucleic acid (DNA) was determined by both the Schmidt-Thannhauser(8) and by the Schneider(9) procedures. The

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TABLE I.
Effect of Herpes Infection on Mass of the Embryo, Embryonic Liver, and Embryonic Heart.

		Liver wt, mg		Heart wt, mg		Embryo wt, g
Tissue	No. of embryos	Dry	Moist	Dry	Moist	Moist
Normal	18-43	34.4	172	12.0	88.3	11.46
Viral	17-36	47.2	245	14.6	118	10.20

TABLE II.
Nitrogen and Moisture Content of Embryonic Tissues Infected with Herpes Virus.

Tissue	No. of embryos	Total* N	Protein* N	Moisture†
Normal Heart	18	11.0	10.2	86.8
Herpetic Heart	17	10.8	10.0	87.4
Normal Liver	22	11.6	10.2	79.5
Herpetic Liver	22	11.8	10.9	80.8

* % of dry weight.
† % of moist weight.

TABLE III.
Succinoxidase and α-Ketoglutaric Acid Oxidase Activities of Embryonic Tissues Infected with Herpes Virus.

		Succinoxidase		α-Ketoglutaric oxidase	
Tissue	No. of embryos	Q_{O_2}*	Total organ†	Q_{O_2}*	Total organ†
Normal Heart	22	12.1	53.5	8.0	36.2
Viral Heart	21	8.8	51.3	6.3	36.5
Normal Liver	18	16.9	154	9.6	87.7
Viral Liver	17	17.6	234	6.9	91.5

* Microliters oxygen per 20 mg moist tissue per 10 min.
† Microliters oxygen per total organ per 10 min.

pentosenucleic acid (PNA) was determined by Schneider's method(9).

Results. Effect of virus on growth. Three days after inoculation of herpes virus, the moist weights of liver and heart are 42 and 34% greater, respectively, than the organs of normal embryos (Table I). These results are in agreement with the findings of Anderson(1). However, in addition to these gross observations, analyses were made which revealed no significant differences in the per cent moisture, protein-nitrogen or total nitrogen of these infected and normal organs (Table II). Furthermore, the number of nuclei per gram of tissue is essentially the same in both normal and infected livers (Table III).

This indicates that after inoculation with herpes, there is an increase in the number of cells, in the dry weight, and in the total protein of the infected organs. In the case of liver it represents approximately a 40% increase in the total protein nitrogen. This accelerated growth rate occurs despite the fact that the total weight of the infected embryo is decidedly less than that of the normal embryo (Table I). The infected tissues appear to develop more rapidly at the expense of the rest of the chick.

Effect of virus on respiratory enzymes. Since the amount of functioning liver available to the embryo is proportionately larger after herpes infection, it was of interest to obtain some measure of the metabolic rate of the organ under these conditions. The activity of some of the respiratory enzymes of the Krebs' cycle has been shown to be lower in regenerating liver(10), and tumor tissue (6,11,12) than in the corresponding normal

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TABLE IV.
Nucleic Acid and Nuclei Content of Normal and Herpetic Embryonic Liver.

Tissue	No. of embryos	Nuclei per g liver*	DNA mg/g tissue†	PNA mg/g tissue†
Normal Liver	18	760×10^6	10.8	29.3
Herpetic Liver	18	863×10^6	13.4	36.2

* Moist wt of tissue.
† Dry wt of tissue.

tissues. The correlations previously reported between changes in the rate of growth of a tissue and the concentration of these respiratory enzymes, suggested the investigation of possible changes in the rate of oxidation of succinic and α-ketoglutaric acids. Our results show that while the succinoxidase activity is not significantly different in the infected and normal livers, there are differences in the succinoxidase activity of normal and herpetic heart tissues. The infected heart was found to have 27% less activity per unit of moist weight than the control tissue (Table III). The α-ketoglutaric oxidase activity per unit of moist weight of infected heart and liver were both found to be lower than that of normal organs by 21 and 29% respectively (Table III).

Effect of virus on nucleic acids. While the total protein of the host cell appeared to be unaffected by the virus infection, the results of the enzyme assays suggest that there might be profound changes in some of the functional proteins which occur in small amounts. Thus to obtain some information concerning the alteration in nucleoproteins, analyses for desoxyribosenucleic acid (DNA) and pentosenucleic acid (PNA) were made. The results of Table IV indicate that there is an increase of 24% in the total concentration of nucleic acid (NA) per unit of dry tissue. The increase in NA is the result of an increase in both DNA and PNA. Thus there is no appreciable change in the PNA/DNA ratio. The propagation of herpes virus appears to induce a marked stimulation in the nucleic acid synthesis of the host cell since the concentration of NA not only increases, but the total mass of the liver increases.

Discussion. Anderson describes herpetic hearts as being edematous, necrotic, enlarged and with lesions. The cells are swollen and the nuclei enlarged. The degree of inflammatory reaction appears to vary with the amount of necrosis produced by the infection (1). In tissue culture Rivers *et al.* find that there is a thickening of the epithelium, giant amitotic cells, and swollen cells(2). These findings would indicate that in the embryo the enlargement of the infected organs is due to an inflammatory reaction and edema. Our observations indicate that the enlargement of the liver and heart is due to an accelerated rate of proliferation which accompanies the herpes infection. This is borne out by the facts that the mass of the herpetic liver is greater than that of the normal, while the percentage composition is the same with regard to moisture, protein nitrogen and total nitrogen. In addition, the number of nuclei found per unit moist weight of tissue is approximately the same in viral and normal liver. There are simply more cells, protein and dry weight in the former than the latter.

The amount of functioning liver and heart in the herpetic embryo is proportionately larger than in the normal because firstly, these organs are larger and secondly, the embryo is smaller. However, the α-ketoglutaric oxidase activity per unit weight of heart and liver was found to decrease to such an extent that the calculated activities of both the total enlarged heart and enlarged liver were no greater than the total activities of the smaller normal organs. This is also true of the succinoxidase activity of the heart. Thus in the herpetic organs there is some loss of metabolic activity which appears to be compensated for by an increase in the size of the organ. The succinoxidase activity per unit weight of liver, however, does not decrease; hence, the total liver succinoxidase available to the embryo increases by nearly 50%.

It is believed that both of these enzymes are localized in liver in the mitochondrial particles of the cell(13,14,15). A measure of their activity in the assay procedures used requires the presence of intact mitochondria in the homogenate. Thus if the loss of α-ketoglutaric oxidase activity in the cell were due to necrosis of the cell and mitochondrial particles, one would expect an equal loss in both respiratory enzymes. The loss of α-ketoglutaric acid oxidase activity relative to the succinoxidase would appear to be a specific effect representing a change in the enzyme pattern of the tissue. The possibility remains that the change in activity might not be in the dehydrogenase enzymes proper but in some part of the hydrogen transport mechanism (which they do not use in common).

One of the most striking changes accompanying the herpetic infection is the accelerated rate of nucleic acid synthesis. The total nucleic acid of the herpetic liver when calculated on the basis of the total dry weight is 70% greater than normal.

There is little change in the ratio of DNA to PNA. If this has any significance with regard to the composition of the virus, as suggested by other workers(16,17,18), it would be that herpes virus contains both DNA and PNA. However, it has also been reported that during that period of embryonic development when protein metabolism is most conspicuous, there is a rise in the concentration of PNA and DNA(19). Thus, the rise in NA might as easily be correlated with the accelerated growth rate of the infected liver.

Summary. 1. A description is given of some of the biochemical changes which occur in tissues infected with herpes virus. 2. Evidence is presented which indicates that herpes virus causes accelerated proliferation in the host cells of embryonic liver and heart. 3. The propagation of the herpes virus appears to induce a marked stimulation in the nucleic acid synthesis of the host cell. 4. It is shown that in herpetic liver there is a change in the normal ratio of succinoxidase to α-ketoglutaric acid oxidase activity. Herpetic infection of the heart is accompanied by a decrease in succinoxidase and α-ketoglutaric acid oxidase activities.

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Response of Man to Egg-Adapted Colorado Tick Fever Virus.* (17830)

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In a previous note(1) the results of immunization of 4 human volunteers with an egg-adapted strain of Colorado Tick Fever

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TABLE I.
Presence of Neutralizing Antibodies in Sera of Volunteers Inoculated with Egg-adapted Colorado Tick Fever Living Virus.

No.	Serum specimen Days post-inoculation	Mortality ratio of mice inoculated intracerebrally with serum-virus dilutions: 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	LD_{50} virus titer	LD_{50} doses neutralized
1	0		6/6	3/5	2/6	0/6			$10^{-3.45}$	140*
	15	6/6	4/6	2/6	0/6				$10^{-2.50}$	1260
	31	5/6	5/6	5/6	2/6	0/6			$10^{-3.40}$	160
	45	6/6	3/6	1/6	0/5				$10^{-2.15}$	2820
2	0			6/6	6/6	6/6	1/6	0/6	$10^{-5.60}$	0
	15		6/6	5/5	3/6	0/5	0/5		$10^{-4.00}$	40
	45		4/4	4/6	0/5	0/4	0/6		$10^{-3.25}$	225
3	0		6/6	4/5	0/6	0/6	0/6	0/6	$10^{-3.40}$	160*
	15	6/6	6/6	2/6	1/6	0/6	0/6		$10^{-2.90}$	500
	31	6/6	4/6	4/6	0/6	0/6			$10^{-3.00}$	400
	45	6/6	3/5	0/6	0/5	0/6			$10^{-2.20}$	2510
4	0			6/6	5/5	5/6	1/5	0/6	$10^{-5.60}$	0
	15		6/6	5/5	3/6	1/5	0/6		$10^{-4.50}$	15
	31	6/6	6/6	3/5	1/6	0/5	0/5		$10^{-3.30}$	200
	45	5/5	1/6	0/6	0/6	0/6			$10^{-1.60}$	10000
10	0				6/6	6/6	2/6	2/6	$10^{-6.00}$	0
	15		5/6	6/6	6/6	1/6			$10^{-4.50}$	30
	47		6/6	6/6	4/6	1/6			$10^{-4.35}$	45
12	0				6/6	4/6	0/6	0/6	$10^{-5.25}$	0
	15		6/6	6/6	6/6	2/6			$10^{-4.75}$	3
	47		6/6	5/6	1/6	1/6			$10^{-3.55}$	50
15	0				6/6	5/6	0/6	0/6	$10^{-5.40}$	0
	15		6/6	5/6	5/5	0/6			$10^{-4.40}$	10
	30		6/6	6/6	5/6	0/6			$10^{-4.40}$	10
	54		6/6	4/6	1/6	0/6			$10^{-3.35}$	110
17	0				6/6	6/6	2/6	1/6	$10^{-5.80}$	0
	15		5/6	6/6	5/7	1/6			$10^{-4.30}$	30
	30		3/5	1/4	6/6	0/6			$10^{-4.10}$	50
	54		2/4	0/6	0/6	0/6			$10^{-2.00}$	6310

* Based on average LD_{50} virus titer of $10^{-5.6}$ in normal serum.

(CTF) virus were reported. In view of the good immunological response obtained in the inoculated individuals and because of the relative innocuousness of the procedure, it was decided to repeat the experiments with a larger group of volunteers.

Strain of virus. The Florio egg-adapted strain of CTF virus was employed(1-3). The inoculum was prepared in the same manner as described previously(1). One batch represented the 37th egg-passage(1) and the other the 74th egg-passage of the virus. Both batches were distributed in lyophilized form. At the time of inoculations enough physiological salt solution was added to make a 10% concentration of the embryo suspension. Prior to inoculation both preparations were tested for their immunogenic potency in mice (1) and were found to give a high degree of protection to mice challenged subsequently with mouse-brain adapted virus. Twenty individuals, 17 males and 3 females 21 to 60 years of age, volunteered for the inoculations. Individuals 1 to 12 received a subcutaneous injection in the region of the biceps muscle of 1.0 ml of a suspension representing the

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TABLE II.
Neutralization Tests on Sera of Volunteers Inoculated with Egg-adapted Colorado Tick Fever Living Virus.

No.	Serum specimen Days post-inoculation	Mortality ratio of mice inoculated intracerebrally with serum-virus dilutions:							LD_{50} virus titer	LD_{50} doses neutralized
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
5	0				5/5	5/5	1/4	0/6	$10^{-5.65}$	0
	47	6/6	6/6	4/6	1/5				$10^{-3.35}$	200
6	0	5/5	6/6	2/6	1/5	0/6			$10^{-2.85}$	1415*
	48	3/4	2/6	1/6	0/6				$10^{-1.75}$	17780
7	0				6/6	4/6	0/6	1/5	$10^{-5.35}$	0
	47	5/5	4/6	4/6	0/6				$10^{-3.00}$	225
8	0				6/6	6/6	4/6	2/6	$10^{-6.50}$	0
	48		6/6	6/6	4/6	1/6	0/5		$10^{-4.35}$	140
9	0				6/6	6/6	4/6	2/6	$10^{-6.50}$	0
	47		6/6	5/5	1/6	0/6	0/6		$10^{-3.60}$	795
11	0			5/5	6/6	2/6	0/5		$10^{-4.75}$	0
	47	6/6	5/6	1/6	0/6				$10^{-2.50}$	175
13	0				6/6	6/6	2/6	1/6	$10^{-5.90}$	0
	56		5/6	5/6	1/6	0/6			$10^{-3.40}$	315
14	0				6/6	6/6	4/6	1/6	$10^{-6.35}$	0
	54		6/6	4/6	0/6	0/6			$10^{-3.25}$	1260
16	0				6/6	6/6	4/6	0/6	$10^{-6.25}$	0
	54		6/6	4/6	2/6	1/6			$10^{-3.60}$	445
18	0				6/6	6/6	5/6	2/6	$10^{-6.65}$	0
	55		6/6	6/6	3/6	0/6			$10^{-4.00}$	445
19	0				6/6	6/6	3/6	2/6	$10^{-6.30}$	0
	54		5/6	2/6	0/5	0/6			$10^{-2.60}$	5010
20	0				6/6	5/5	2/6	0/6	$10^{-5.75}$	0
	54		6/6	3/6	2/6	0/6	0/6		$10^{-3.30}$	280

* Based on average LD_{50} virus titer of $10^{-6.00}$ in normal serum.

37th egg-passage, whereas individuals 14, 16, 19 and 20 were given subcutaneously 1.0 ml and volunteers 13, 15, 17 and 18 received 0.1 ml intradermally of the 74th egg-passage material. Blood specimens were secured from all of the individuals prior to and at various intervals after the inoculations.

Neutralization tests. Serum samples obtained from the individuals before and after inoculations were submitted to neutralization tests against mouse-brain adapted Condon(2) strain of CTF virus. The only deviation in the technique described previously(1) was that the serum-virus mixtures were incubated for 26 hours prior to inoculation in mice instead of 2 and 26 hours.

Results. Clinical observations. Of the 12 individuals (1 to 12) who were inoculated with the 37th egg-passage material, 2 (nos. 3 and 6, Tables I and II) had a history of a prior laboratory infection with CTF virus(4) and one (no. 1, Table I) had lived in an endemic area for many years. These 3 individuals showed no clinical reaction whatsoever to the inoculation. The remaining 9 individuals in this group developed enlarged, tender axillary lymph glands on the side of the injection which subsided within 2 to 7 days after inoculation. Four of these volunteers had a mild attack of an illness which

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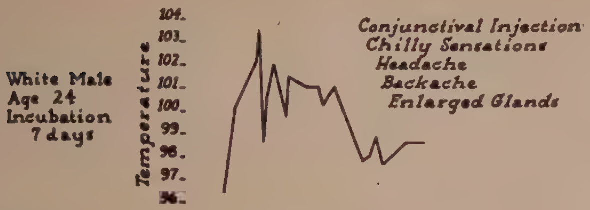

Days	1	2	3	4	5	6	7
Leucocytes in Thousands per c.mm.	6.55	3.63	3.08	4.08	4.65		7.22
Segmenters %	58	39	29	24	37		47
Band Forms %	10	30	25	23	15		4
Lymphocytes %	19	24	36	38	39		45
Monocytes %	11	7	10	14	8		3
Eosinophils %	1			1	1		1
Basophils %	1						

FIG. 1.
Clinical findings in a volunteer inoculated with the egg-adapted strain of Colorado tick fever virus.

lasted 2 to 5 days and resembled CTF except for the following aspects. The onset was 6 to 7 days after inoculation, as compared to 3 to 4 days in the individuals infected with "human" or "hamster" passaged strains of CTF virus(4). There was a single instead of a dual episode of illness and the onset and disappearance of symptoms were gradual, rather than relatively abrupt. In each of the 4 individuals a low white blood cell count and differential blood picture characteristic of CTF was found(4). Three of the volunteers (nos. 2, 4 and 8), 2 of whom had reacted with enlarged lymph glands and 1 who had become ill after the inoculation, were submitted to intravenous challenge inoculations with a human strain of CTF virus 10, 6 and 6 months, respectively, after inoculation with the egg-passage material. No signs of illness were noted in any of these 3 individuals following the challenge inoculation with the human strain.

In the second group of 8 volunteers (13 to 20) inoculated with the 74th egg-passage virus, all became ill after an incubation period of 6 to 7 days, and 7 had enlarged lymph glands near the site of injection. The disease lasted from 3 to 4 days and again only a single attack occurred in contrast to the typical CTF infection(4,5). As in the previous group, the onset and subsidence of symptoms were gradual but the course of the disease seemed to be more severe, particularly in those volunteers who had been inoculated by the intradermal route. Fig. 1 depicts the clinical findings in a volunteer of this group which may be compared to the findings in Fig. 2 of a typical case of naturally acquired CTF infection. Volunteers 13 and 19 (Table II) were challenged by the intravenous route with a human strain of CTF virus 3½ and 5 months, respectively, after immunization with the egg-passage virus. As with the 3 individuals challenged in the first group of volunteers, no signs or symptoms of illness were observed following the challenge inoculation.

Serological data. In order to determine, on a preliminary basis, when the highest level of circulating antibodies would be encountered

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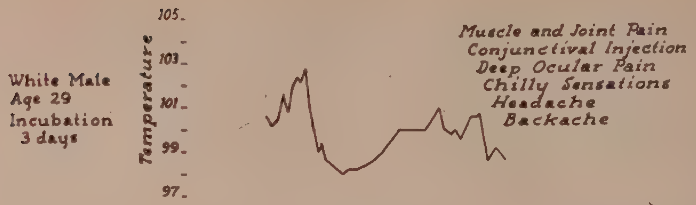
5. Cox, H. R., Colorado Tick Fever, Chap. 27, Viral and Rickettsial Infections of Man, edited by T. M. Rivers. J. B. Lippincott Co., Philadelphia, 1948.



Days	1	2	3	4	5	6	7	8	9	12
Leucocytes in Thousands per c.mm.	8.30	3.23	4.65	3.40	4.23	2.30	2.55	2.88	3.40	7.30
Segmenters %	58	59	25	21	25	19	19	15	16	39
Band Forms %	4	11	20	20	22	32	37	28	31	6
Lymphocytes %	31	18	44	47	40	40	37	45	45	51
Monocytes %	2	10	11	11	12	7	7	12	6	3
Eosinophils %	4	2		1	1	1			2	1
Basophils %	1					1				

FIG. 2.

Clinical findings in a typical case of naturally acquired Colorado tick fever infection.

after immunization, sera from 6 individuals of the first group and from 2 individuals in the second group were submitted to neutralization test. It is observed in Table I that the pre-inoculation sera obtained from individuals 1 and 3 showed significant neutralizing properties when compared to the pre-inoculation sera of the other volunteers, who showed an average LD$_{50}$ virus titer of $10^{-5.6}$. This is not surprising since individual 3 had become infected previously in the laboratory with CTF virus and volunteer 1 had lived in an endemic CTF area. Antibody titers determined on the 15th and 31st post-inoculation days indicated that these 2 individuals showed a somewhat more rapid serological response, a fact compatible with their previous history. All volunteers, regardless of previous history, showed their highest levels of neutralizing antibodies at the 45th to 54th day after inoculation. The serological response varied, however, from individual to individual, as shown by the fact that serum samples from 10 and 12 neutralized only 45 and 50 LD$_{50}$ of virus, whereas serum samples from 4 and 17 neutralized 10,000 and 6,310 LD$_{50}$ of virus, respectively.

In view of the fact that the serum samples obtained 45 to 54 days after inoculation seemed to contain more antibodies than the samples obtained at shorter intervals after inoculation, it was decided to submit only 2 serum samples from each individual to the neutralization test; one pre-inoculation specimen and one obtained at 47 to 56 days after inoculation. It may be observed in Table II that the pre-inoculation serum of individual 6 showed significant neutralizing antibodies when compared with the sera of the other volunteers, who showed an average virus titer of $10^{-6.0}$. Since volunteer 6 had contracted a laboratory infection with CTF virus prior to the inoculation, this enhanced serological response conforms with the history of prior infection. The serological response of all of the other volunteers was good, with the possible exception of individual 8, as judged by the neutralization index of 100 or more obtained with the serum samples after inoculation.

From 3 of the 5 volunteers who had been challenged with the human strain of CTF virus, serum samples were available for determining their neutralizing potency. It may be seen in Table III that the sera obtained after the challenge inoculation indicate a

TABLE III.
Comparison of Neutralization Titers in Sera of Three Volunteers Inoculated with Egg-adapted Colorado Tick Fever Living Virus and Challenged with Human Strain of Colorado Tick Fever Living Virus.

No.	Serum specimen Days post-inoculation	Mortality ratio of mice inoculated intracerebrally with serum-virus dilutions: 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	LD_{50} virus titer	LD_{50} doses neutralized
2	0			6/6	6/6	6/6	1/5	0/6	$10^{-5.65}$	0
	31			6/6	6/6	5/6	1/6	0/6	$10^{-4.50}$	15
	20 days after challenge*	5/5	3/6	3/6	0/6	0/6			$10^{-2.50}$	1415
8	0†				6/6	6/6	3/6	0/6	$10^{-6.00}$	0
	15			6/6	6/6	6/6	2/5	1/5	$10^{-5.75}$	2
	64 days after challenge‡	6/6	1/6	1/6	0/6	0/6			$10^{-1.70}$	19950
19	0†				6/6	6/6	3/6	0/6	$10^{-6.00}$	0
	31		6/6	6/6	3/6	0/5	0/6		$10^{-4.00}$	100
	65 days after challenge§	1/6	0/6	0/6	0/6				$<10^{-1.00}$	>100000

* Challenged with human strain 10 months after inoculation.
† Pre-inoculation serum sample of volunteer 20 used because of insufficient amount of serum.
‡ Challenged with human strain 6 months after inoculation.
§ Challenged with human strain 5 months after inoculation.

marked increase in their neutralizing indices and show neutralizing antibody titers as high or higher than those shown in Tables I and II. Since the volunteers were not bled immediately prior to the challenge inoculation, it cannot be assumed with certainty that the rise in circulating antibody occurred solely as a result of the challenge inoculation. On the basis of the results summarized in Tables I and II which show that the serological response of normal individuals increased with the duration of time following inoculation, it is apparent that this fact must be considered also before attributing the higher neutralizing values to the challenge dose.

Summary. Twenty volunteers were inoculated with the egg-adapted strain of CTF living virus. Reactions ranged from enlarged, tender axillary lymph glands to single-episode attacks of illness resembling Colorado Tick Fever. Five of the inoculated individuals were subsequently challenged with a human strain of CTF living virus but they showed no reactions whatsoever to this second inoculation. All of the individuals developed neutralizing antibodies, but the 3 who presumably were immune prior to the inoculations showed a comparatively earlier and greater response. Sera obtained from the 3 individuals after they had been challenged with a human strain likewise showed a high neutralizing titer.

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Further Studies on Toxicity of Thioacetamide in Rats. (17831)

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In a previous study(1) we have shown that weanling albino rats placed on a diet containing 0.032% thioacetamide (TA) for 190 days showed changes in the liver characterized by irregular fibrosis, hyperplasia of the bile ducts, fatty infiltration, necrosis and nodular regeneration of the parenchymal cells. Since it is a well established fact that diffuse hepatic fibrosis is a regular occurrence not only in rats on a diet low in lipotropic factors, but also after the administration of toxic substances which may undergo reversal by lipotropic factors or by dietary means(2,3,4,5,6,7,8), studies were undertaken to determine the effect of lipotropic factors on the course of the lesions in the liver produced by the ingestion of thioacetamide.

The present report is concerned with: (a) studies on spontaneous regression after discontinuance of the hepatotoxic agent, (b) determination of the onset of liver necrosis, and (c) the prophylactic effect of lipotropic factors (choline, methionine, and casein).

Experimental methods and results. Since, as reported previously(1), fatty infiltration and fibrosis with nodular regeneration were found in the livers of rats on a diet containing as little as 0.032% TA, the present studies were conducted at this dietary level of intake. The diet to which TA was added was the same as that previously used (Purina Dog Chow). Both male and female weanling rats were used. The livers from 5 rats maintained on 0.032% TA for 120 days and later placed on the basic diet alone for 170 days showed no spontaneous regression. Grossly the livers of these rats appeared mottled (uniformly nodular) and yellowish green. Since these livers appeared nodular on gross observation, no histological studies were made. Rats maintained on 0.032% TA for 30, 60, and 120 days showed varying degrees of liver damage. The first effect observed in the livers of 3 rats on the diet for 30 days was to bring about cellular hypertrophy with relative increase in size of the nucleus and nucleolus. This effect seemed to be confined to the liver parenchymal cells. At the same time that these hypertrophic changes were occurring in the liver cells a few were apparently succumbing to the effect of TA, since their nuclei were missing and the cytoplasm lacked definition. By the 60th day most of the livers from 4 rats showed some scarring, although collagen was not abundant. In the 6 rats on the diet for 120 days, many, perhaps half, of the cells did not show the hypertrophic change though the nucleoli remained relatively prominent. The hypertrophied cells tended to occur in clusters, as described previously in rats on the diet for 190 days (1).

In the experiments that follow, dietary supplements known to have lipotropic action were added to the basic diet (Purina Dog Chow) containing 0.032% TA as follows: Diet 1, Basic diet + 0.032% TA; Diet 2, Diet 1 + 0.7% DL-methionine + 2% yeast; Diet 3, Diet 1 + 30% casein; Diet 4, Diet 1 + 0.2% choline chloride + 2% yeast; Diet 5, Basic diet alone.

The rats were kept on the respective diets

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TABLE I.
Statistical Comparison of Livers of Rats Receiving Thioacetamide with and without Lipotropic Factors. 5 Rats/Group.

Diet		Liver wt		
		% of bd. wt	S*	e*
1	Basic + 0.032% TA	7.39	.77	.35
2	No. 1 + 0.7% DL-Methionine + 2% Yeast	5.39	.08	.03
3	No. 1 + 30% Casein	5.80	.37	.17
4	No. 1 + 0.2% Choline Chloride + 2% Yeast	5.57	.35	.15
5	Basic (Control)	3.22	.20	.09

* S—Standard deviation. e—Standard error of the mean.

for 78 days, after which time they were weighed, autopsied, and the liver carefully removed and weighed. Grossly the livers from rats on Diets 1, 2, 3, and 4 were indistinguishable, with perhaps the livers from rats on Diet 3 appearing slightly less affected than the livers of rats on the other three diets.

In the table, data are given on the mean percentage of the liver weight to body weight for each group of rats. Based on the relationship of liver weight to body weight, the livers of rats on diets containing no lipotropic agent (Diet 1) other than that not known to be present in the diet, and those on 30% casein (Diet 3) were the heaviest, while those on the diets containing DL-methionine (Diet 2), and choline (Diet 4) were the lightest. However, even these livers were heavier than those of rats on the basic diet (Diet 5) alone. The smaller size of the livers of rats given the active lipotropic agents, DL-methionine and choline (Diets 2 and 4), respectively, is significant and is probably related to the amount of fat present. Histopathological study of the livers of rats on the four different dietary regimens containing 0.032% TA showed that the most severe changes occurred in rats on Diet 2, with the least severe changes in rats on Diet 3. Females on the latter diet showed advanced changes.

Since liver fibrosis, nuclear and cytoplasmic inclusion, and cell variability were observed in all of the rat livers, except those on Diet 5, it cannot be said that any of the dietary lipotropic supplements were effective in preventing liver damage, although there appears to have been produced a quantitative alteration in the character of the lesion.

Histological changes of tissues were not remarkable except for the thyroids. In all animals, other than the controls, the thyroids appeared grossly atrophic. On histological examination, the thyroid alveoli contained dense colloid and the lining epithelial cells were flattened, suggesting inactivity.

Conclusions. As has been reported by Orr (6) and by Sellers *et al.*(7) spontaneous regression of liver cirrhosis may occur on discontinuance of the hepatotoxic agent when damage to the liver is not too severe. In our studies rats maintained on 0.032% TA for 120 days and then placed on the basic diet alone for 120 days showed no regression in the amount of liver damage produced by TA. This is in agreement with the findings of Cameron and Karunaratne(8) that the cirrhosis produced after the prolonged administration of carbon tetrachloride is irreversible.

Rats on 0.032% TA for 30 days all showed some changes in the liver which became progressively worse after 60 and 120 days on the diet. The addition of the active lipotropic agents (choline, DL-methionine, or casein) to the diet containing 0.032% TA did not prevent liver damage.

Summary. Scarring and nodular hyperplasia of the liver were produced in rats maintained on an otherwise adequate diet containing 0.032% thioacetamide (TA). Administration of lipotropic factors (choline or DL-methionine and casein) in conjunction with 0.032% TA significantly decreased liver weight when compared to body weight. On the other hand histological examination of the livers from rats on the diet containing 30% casein in conjunction with 0.032% TA

(Diet 3) showed the least severe changes, while the livers of rats on Diet 2 (DL-methionine) showed the most severe changes. Since fibrosis and various degrees of parenchymal lesions were observed in all rats on 0.032% TA with and without the added lipotropic factors, it cannot be said that any of the lipotropic factors studied were effective in preventing liver damage, although there appears to have been a quantitative alteration in the character of the lesion.

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Failure of Malononitrile in Therapy of Experimental Poliomyelitis.*
(17832)

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Recently Szanto and Felsenfeld(1) reported malononitrile to be effective in protecting more than fifty per cent of mice infected with a low concentration of the Lansing strain of poliomyelitis and prolonging the life span of mice infected with a larger dose of the virus. They also reported that mice treated with malononitrile after onset of paralysis showed a markedly prolonged survival time, about one-third of which recovered completely or partially. The purpose of this communication is to report the failure of malononitrile to protect mice against intracerebral inoculation of the Lansing strain of poliomyelitis. It was also found that this drug had no therapeutic effect when administered to mice after the onset of paralysis.

Methods. The Lansing strain of poliomyelitis virus was used in all therapy experiments. The technic of preparation and storage of mouse brain suspensions of this virus for intracerebral inoculation is described elsewhere(2). The identity of the Lansing strain used in this laboratory is periodically verified by production of typical paralysis in rhesus monkeys following intracerebral inoculation, histopathologic findings and neutralization tests with hyper-immune Lansing antiserum. Two strains of Swiss mice (Webster & Beyer) weighing 13 to 15 g were employed in the present studies. Groups of mice were inoculated with either 10 or 100 LD_{50} units of virus intracerebrally in chemotherapy experiments. The LD_{50} titer of the virus was approximately $10^{-3.5}$. Two types of experiments were done. In the first, mice were inoculated daily with malononitrile $CH_2(CN)_2$[†] beginning 24 hours before virus inoculation and therapy was continued until onset of paralysis. In the second, mice were treated with malononitrile after onset of paralysis, and the survival time was compared with that of untreated, paralyzed mice. The survival time of the paralyzed mice was recorded as the time when the mice were found dead. In most instances the actual time of death was observed.

Results. Mice were inoculated intraperitoneally with 2.5 to 3 mg per kg body weight daily in therapy experiments. The dosage of 3 mg per kg of body weight was given in most experiments, and we also found that this amount of drug was well tolerated by the mice. Combined results obtained in 3 experiments with groups of treated and control mice challenged with approximately 10 and

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* Aided by a grant from the Michael Reese Research Foundation.

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[†] Obtained from the Schwarz Laboratories, N.Y.

TABLE I.
Results Obtained in Protection of Infected Mice with Malononitrile.

LD_{50} dose	No. of mice	Drug given	Avg incubation period, days	% mortality
10	32	+	12.0 ± 3.8	80
10	38	0	15.9 ± 5.3	76.4
100	32	+	11.3 ± 2.5	94.6
100	38	0	11.1 ± 3.2	97.7

TABLE II.
Results Obtained in Therapy of Paralyzed Mice with Malononitrile.

LD_{50} dose	No. of mice	Drug given	Avg survival time after onset of paralysis in hr
10	29	+	32.5 ± 14.6
10	30	0	32.1 ± 10.2
100	25	+	29.3 ± 14.5
100	21	0	31 ± 11

100 LD_{50} units of virus intracerebrally are shown in Table I. Malononitrile was started 24 hours before virus inoculation and continued daily until onset of paralysis. About half of the treated mice received the drug daily for at least 10 days prior to onset of paralysis. The results shown in Table I indicate that there was no significant difference in average incubation period or mortality rate in the treated and control mice challenged with 10 or 100 LD_{50} units of virus respectively.

Table II summarizes the results obtained when 3 mg per kg body weight of malononitrile was given after onset of paralysis in mice initially challenged with 10 and 100 LD_{50} units of Lansing virus. Again no significant difference was observed in survival time between the control and treated groups of animals. All mice which developed paralysis uniformly succumbed regardless of malononitrile therapy.

Discussion. The identity of the virus employed in the malononitrile therapy experiments described by Szanto and Felsenfeld(1) is open to question because of the high M.L.D. titer described (3.2×10^{-7}) and the relatively short survival time after onset of paralysis in their control mice. Furthermore, these authors describe no attempts to check the identity of the virus strain that they employed in order to rule out the possible hazard of a spontaneous mouse neurotropic virus. In our experience and that given in the literature(3,4) the LD_{50} titer of the Lansing strain in infected mouse CNS tissue usually ranges from $10^{-3.4}$ to $10^{-4.2}$ in Swiss mice inoculated intracerebrally.

We feel that adequate dosages of malononitrile were given in our experiments because therapy was started 24 hours before virus inoculation and continued daily until onset of paralysis. We also employed the recommended dosage per kg body weight in most of our experiments. On the other hand, Szantos and Felsenfeld gave the drug the day after infection and started daily administration 6 days later for 10 consecutive days in mice inoculated with a low M.L.D. of virus. Mice inoculated with a high M.L.D. of virus were started on therapy the day after infection.

Summary. Malononitrile was found to have no beneficial effect in either the protection or therapy of mice infected with the Lansing strain of poliomyelitis.

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Cultivation of Poliomyelitis Virus (Lansing Strain) in Human Embryonic and Placental Tissues.* (17833)

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Recently Enders *et al.*(1,2) have reported the successful cultivation of the Lansing strain of poliomyelitis virus in human embryonic and foreskin tissues. These workers presented the first evidence that the poliomyelitis virus is capable of *in vitro* multiplication "in cells other than those of nervous origin." Prior to these reports Sabin and Olitsky(3) demonstrated multiplication of a monkey-adapted strain of poliomyelitis (MV) in tissue cultures composed of human embryonic brain fragments. Their attempts to propagate this strain in non-nervous tissues were unsuccessful. Although various strains of mouse encephalomyelitis virus multiply readily in animal tissue cultures (4,5) and the chick embryo(6), experiments with the poliomyelitis virus have thus far given negative results.

The present studies were undertaken to confirm the results of Enders *et al.*(1) on the cultivation of the Lansing strain of poliomyelitis in human embryonic tissues. Experiments were also carried out to attempt propagation of the Lansing strain in placental tissue because of the difficulty in obtaining human embryos.

Methods. The technic of Weller and Enders(7) was employed in the preparation of all tissue cultures. Essentially the cultures consisted of tissue fragments suspended in 3 to 20 cc of a mixture of balanced salt solution (3 parts) and ox serum ultrafiltrate (1 part). Tissues of human embryos ranging from 4 weeks to 4 months of age and premature infants approximately 7 months of age were used. Most of the tissues were obtained from embryos 2 to 4 months old. The following tissue fragments were employed: skin, intestine, arm and leg muscle and brain. Both individual and pooled tissue cultures were prepared. The initial inoculum of each tissue culture flask consisted of 0.1 to 0.4 cc of a 10% centrifuged mouse brain suspension of the Lansing strain of poliomyelitis virus(8) depending on the volume of nutrient fluid present. The nutrient fluid was removed as completely as possible and replaced at intervals ranging from 4 to 7 days The presence of virus in the supernatant fluid of the tissue culture was demonstrated by inoculation of Swiss mice (Webster and Plymouth strains) and occasionally rhesus monkeys intracerebrally. Penicillin and streptomycin mixtures (250 units/cc) were added to the tissue cultures to prevent bacterial contamination. If contamination occurred, the cultures were discarded. Tissue cultures were also prepared in the same manner from the chorion, amnion and spongios tissues of both mature and immature human placentas. Cultures were composed of both individual and pooled tissues. The identity of the virus used as the primary inoculum and cultivated was verified by (1) production of typical paraly-

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TABLE I.
Multiplication of Poliomyelitis Virus in Various Individual Human Embryonic Tissue Cultures.

Exp. No.	No. supernatant fluid changes	Tissue culture				
		Skin	Intestine	Arm muscle	Leg muscle	Brain
I	3	4/5*	1/6	5/6	1/6	—
	4	5/5	0/5	4/5	1/5	—
	5	3/5	0/6	5/6	1/6	—
II	3	1/6	0/5	0/5	6/6	—
	4	2/6	0/5	1/6	4/6	—
	5	1/6	0/5	0/5	4/6	—
III	3	1/6	—	2/6	0/6	3/5
	4 (contaminated)					
IV	3	2/5	—	2/4	—	—
	4	2/6	—	2/5	—	—
	5	3/5	—	2/5	—	—
V	3	1/5	0/4	1/4	—	0/5
	4	2/4	0/5	0/5	—	0/5
	5	1/5	0/4	0/4	—	0/5
VI	3	4/4	0/4	—	0/6	—
	4	2/4	0/4	—	0/6	—

* Numerator = No. of mice which developed paralysis.
Denominator = No. of mice inoculated.

sis in mice and rhesus monkeys following intracerebral inoculation, confirmed by histopathology and (2) neutralization tests with hyperimmune Lansing monkey antiserum.

Results. Evidence of virus multiplication was obtained in 9 of 16 embryos in various tissue cultures consisting of both pooled and individual tissues. In most of the positive pooled tissue cultures virus was demonstrated in the fifth nutrient fluid supernatant change which represents an approximate dilution of the original inoculum of 10^8 times. The LD_{50} titer of the original mouse brain inoculum was approximately $10^{-3.5}$. In another experiment virus was demonstrated in the third supernatant of a transfer of the original culture (5th supernatant) to a second fresh tissue culture representing an approximate dilution of 10^{11} times of the original inoculum.

Two rhesus monkeys inoculated intracerebrally with the third supernatant fluid change of a pooled tissue culture consisting of skin, arm muscle, intestine and brain developed a triplegia and paraplegia after an incubation period of 6 and 14 days respectively. These animals were sacrificed and sections of their cervical and lumbar cord showed pathologic findings typical of poliomyelitis. Neutralization tests were conducted with the fourth supernatant of the same tissue culture. Mice inoculated with tissue culture plus hyperimmune monkey Lansing antisera were protected while control mice developed typical paralysis and died. We have also successfully immunized mice by repeated intraperitoneal inoculations of the active tissue culture virus to subsequent intracerebral challenge inoculation with mouse CNS virus. The degree of such active immunity from the tissue culture virus was considerably less than that usually achieved by mouse CNS virus(8).

Virus multiplication was demonstrated in skin tissue cultures in 6 of 6 attempts; in arm muscle in 5 of 5 attempts; in leg muscle in 2 of 4 attempts; in intestine in 1 of 4 attempts and in brain in 1 of 2 attempts. These results are summarized in Table I.

Attempts were also made to propagate the Lansing strain in tissue cultures prepared from the chorion, amnion and spongios tissues of 11 mature and two immature human placentas. The placenta tissue fragments were washed 3 to 12 times with nutrient fluid prior to use in order to remove as much gamma globulin as possible. Evidence for virus multiplication was obtained in only 2 instances. One was a pooled chorion and amnion tissue culture in which virus was demonstrated in the third supernatant (original virus dilution approximately 10^5 times) and the other a spongios tissue culture in the fourth supernatant (original virus dilution approximately 10^7 times). Seven attempts to cultivate the Lansing strain in human foreskin tissue cultures have given negative results, although Weller *et al.*(2) have recently reported positive findings. We feel that our experiments are inconclusive since the foreskins were not received in satisfactory condition and merthiolate was used to disinfect the prepuce prior to circumcision. Additional experiments are planned with more suitable preparations.

Comment. Our findings definitely confirm those of Enders *et al.* on the cultivation of the Lansing strain of poliomyelitis in various human embryonic tissue cultures. We were also able to confirm their claim that this virus is capable of multiplying in cultures composed of non-nervous tissues. The multiplication of the poliomyelitis virus in tissue culture is extremely important because it opens up the possibility of employing tissue culture virus for attempts to produce a poliomyelitis vaccine for human immunization. Undesirable reactions due to inoculation of brain tissue vaccines could thus be eliminated.

Evidence of virus multiplication in placental tissue cultures was obtained in only 2 of 13 experiments. Perhaps the failures were due to the presence of traces of gamma globulin in placental tissues even though these tissues were washed repeatedly with nutrient fluid. The difficulty in removing all antibodies from cells by repeated washings is a common laboratory experience. Moreover, gamma globulin has been shown to have a relatively high neutralizing antibody titer for the Lansing strain of poliomyelitis virus(9,10). Possibly no Lansing neutralizing antibodies were present in the two placenta tissue cultures giving positive results.

Summary. The propagation of the Lansing strain of poliomyelitis virus in various human embryonic tissue cultures was confirmed. The virus was also shown to multiply in cultures composed of non-nervous tissues. Optimum results were obtained with embryonic skin and muscle tissues. The identity of the virus cultivated was established by intracerebral inoculation of mice and rhesus monkeys, histopathologic findings, neutralization tests with Lansing hyperimmune serum, and production of specific active immunity. Evidence of virus multiplication in human placental tissue was obtained in only 2 of 13 tissue cultures. It is suggested that the negative results may have been due to the presence of gamma globulin in the placental tissues.

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10. Levinson, S. O., Milzer, A., and Wolf, A. M., Abraham Levinson, Anniversary Vol., Studies in Pediatrics and Medical History, New York; Froben Press, 1949, p. 131.

Received April 13, 1950. P.S.E.B.M., 1950, v74.

Distribution of Azo-Proteins in the Tissues of the Normal Mouse.* (17834)

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The preparation of a highly colored azo-protein, R-salt-azo-benzidine-azo-ovalbumin (1) provided a chemically labelled antigen which could be detected colorimetrically in

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[*] This paper constitutes part of a report submitted for The Borden Undergraduate Research Award in Medicine at New York Univ. College of Medicine.

1. Heidelberger, M., Kendall, F. E., and Soo, C. M., *J. Exp. Med.*, 1933, v58, 137.

TABLE I.

Code	Protein	mg/cc	Color
R-OA	R-salt-azo-benzidine-azo-ovalbumin	2	Red
S-OA	Safranin-azo-safranin-azo-ovalbumin	2	Purple
AB-OA	Aniline blue-azo-ovalbumin	2	Blue
R-Gel	R-salt-azo-benzidine-azo-gelatin	4	Red
R-C	R-salt-azo-benzidine-azo-casein	1.7	,,
AB-C	Aniline blue-azo-casein	1.7	Blue
AR-C	Alizarin red S-azo-acriflavine-azo-casein	3.5	Purple-red
AC-C	Acriflavine-azo-casein	2	Brown
NR-C	Neutral red-azo-casein	1.5	Brown-red
NRT-C	Neutral red azo-thionine-azo-casein	1.5	Purple

very minute quantities. Several investigations utilizing similarly labelled proteins have since been made to study the distribution of antigenic substances in animal tissues(2-5).

The experiments reported here were undertaken to determine, if possible, the specificity of tissue localization of parenterally administered proteins in normal animals by means of chemical labels which could be detected optically.

Materials and methods. The azo-proteins in Table I were prepared according to the method described(1) for the preparation of R-salt-azo-benzidine-azo-egg albumin.

In preparing the casein group of azo-proteins, 0.17 g of the aromatic amine or diamine in 100 cc water containing 1.5 cc concentrated HCl was diazotized at 5-7°C with 0.09 g $NaNO_2$ and then poured into 125 cc water containing 3.0 g of sodium acetate. 112 cc of the resulting solution was coupled directly to 0.5 g casein in the preparation of AC-C, AB-C, and NR-C, but in the preparation of the other azo caseins, 0.17 g of Neutral Red, 0.23 g Alizarin Red S, or 0.22 g R-salt in 100 cc water was added to the sodium acetate-tetrazonium solution and coupling aided with the addition of 2N Na_2CO_3. 160 cc of the diazonium-azo solution thus prepared was coupled in turn to 0.5 g casein dissolved in 250 cc water at room temperature; coupling was completed with 2N Na_2CO_3.

The R-salt-azo-benzidine-azo-gelatin was purified by acidification with acetic acid and subsequent precipitation with acetone. All azo-proteins were precipitated and redissolved 2 to 4 times and dialyzed against normal saline. These procedures were carried out at 2-5°C. All mice were sacrificed by exsanguination and the tissues were fixed overnight in 12% formalin containing 5% acetic acid. Bouin's fixative or Helly's fluid were used as controls. Gross observations of the distribution of color were made during the procedure of cutting sections of the tissues embedded in paraffin blocks. Sections were counterstained for microscopic study with Mayer's hematoxylin except those containing blue azoprotein and these were left unstained.

Results. Table II gives the gross and microscopic observations made following the injection of groups of mice intravenously with 0.5 cc of several R-salt-azo-benzidine-azo-proteins and disafranin-diazo-ovalbumin. Observation of the presence of azo-protein in the lymph nodes was complicated by the finding that, of several mesenteric and aortic lymph nodes, only one might reveal dye-protein in the fixed macrophages. At the time of these experiments, only a sampling of the mesenteric and aortic nodes was taken from each group of mice. The bone marrow (represented by sections of the femur and sternum) and spleen took on a dark brown color after fixation and, consequently, gross detection of the azo-proteins in these organs was almost impossible.

From the data in Table II, it will be noted that certain differences were observed in the distribution of the various R-salt-azo-pro-

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2. Smetana, H., and Johnson, F. R., *Am. J. Path.*, 1942, v18, 1029.

3. Sabin, F. R., *J. Exp. Med.*, 1939, v70, 67.

4. Pratt, H. N., and Gregersen, M. I., *J. Immunol.*, 1941, v40, 163.

5. Kruse, H., and McMaster, P. D., *J. Exp. Med.*, 1949, v90, 425.

TABLE II.*
Sites of Localization of Some R-salt-azo-proteins and Disafranin-diazo-ovalbumin Following Intravenous Injection into Mice.

No. of mice	Azo-protein	Vol., cc	Time interval	Gross								Microscopic								
				Liver	Kidney	Lungs	Intestine	Heart	Muscle	Nodes	Urine	Liver	Kidney	Lungs	Spleen	Intestine	Heart	Muscle	Nodes	Bone Marrow
3	R-Gel	.5	10 min	0	tr	0	0	0	0	0	+	0	0	0	0	0	0	—	0	—
3	R-C	.5	10 ”	0	3	0	0	0	0	0	+	0	x	0	0	0	0	—	0	—
5	R-OA	.5	20 ”	2	tr-2	0	0	0	0	2	—	2	x	0	0	0	0	T	4	0
4	”	.5	50 hr	3	tr	0	0	0	0	1	—	4	0	0	1	0	T	T	2	0
3	Dilute R-OA (1:5)	.5	30 min	1-2	0	0	0	0	0	—	±									
3	S-OA	.25	30 ”	3	0	1	0	0	—	0	—	2	0	1	0	0	0	—	0	—

(—) Means no observation made.

* The gross observations are recorded arbitrarily from 0 to 4, depending upon the relative intensity of color characterizing the azo-protein: O = no color, 1 = definite but light color, 4 = very intense color, 2 and 3 indicating intermediate grades, tr = trace. Microscopic observations indicate the relative number of cells containing the azo-protein: T = a rare cell, 1 = one macrophage per H.P.F., 2 = one to several macrophages per H.P.F., 3 = almost all the macrophages per H.P.F., 4 = all the macrophages per H.P.F. With respect to the kidney, x indicates observation of color in the tubules; + indicates detection in the urine.

teins though the chemical tag common to them was the same.

In order to answer the question of the importance of the azo-grouping in determining the distribution of the labelled proteins, a series of azo-caseins were prepared and injected intravenously into small groups of mice. The mice were sacrificed an hour after the injections. The results are recorded in Table III.

The possibility of rapid destruction of neutral red-azo-casein, neutral red-azo-thionin-azo-casein and alizarin red-S-azo-acriflavine-azo-casein was then considered, since the failure to detect these azo-proteins in the above distribution study may have been due to this rather than to lack of tissue localization during the time period allowed. Therefore, 6 groups of 2 mice each were injected intraperitoneally with 0.5 cc of the following azo-proteins: AB-C, AC-C, NR-C, NRT-C, AR-C and R-Gel; the mice were sacrificed 28 hours later. The mesentery of each mouse was rinsed with saline, stretched on a glass slide, fixed in formolacetic, stained with Mayer's hematoxylin, and finally mounted in balsam. In each case, the azo-protein was found in large quantities in the macrophages. It became apparent that the macrophages, given a sufficient length of time and suitable conditions, were capable of taking up NR-C, NRT-C, AR-C and R-Gel; failure to detect these azo-proteins in the previous distribution studies, therefore, was not due simply to destruction of the azo-protein.

It will be noted from Table III that differences in the isoelectric points of this series of azo-caseins could hardly be considered sufficient to account for the differences in distribution.

Discussion. The passage of azo-proteins through the mouse kidney was first noticed by Smetana and Johnson(2) and the proteins they used included R-salt-azo-benzidine-azo-casein. These observations seem to be substantiated by the experiments reported here. On the other hand, Pratt and Gregersen(4) reported that they were unable to detect R-salt-azo-benzidine-azo-ovalbumin in the urine of rabbits after intravenous

TABLE III.*

Sites of Localization of Various Azo-caseins and R-salt-azo-gelatin Following Intravenous Injection into Mice. Time interval 1 hour.

				Gross										Microscopic		
No. of mice	Azo-protein	Amt, mg	Approximate isoelectric point, pH	Liver	Kidney	Lungs	Intestine	Heart	Brain	Muscle	Uterus	Nodes	Urine	Liver	Kidney	Lungs
2	AB-C	.85	4.1–4.1	3	tr	0	tr	0	0	tr	tr	3	±	3	0	0
3	AC-C	1.0	4.4–4.2	4	0	0	0	0	0	0	0	tr	0	4	0	0
3	R-C	.85	4.2	1	4	0	tr	0	—	—	—	1	++++	1	x	0
4	AR-C	1.75	4.6–4.5	0	0	0	0	0	0	0	0	0	+	0	0	0
3	NR-C	.75	4.5–4.5	tr	0	0	0	0	0	0	0	0	0	0	0	0
3	NRT-C	.75	4.5	0	0	0	0	0	0	0	0	0	0	0	0	0
3	R-Gel	2.0	—	1	1	0	tr	0	—	—	—	tr	+	0	0	0

* Symbols same as for Table II.

administration.

The smallest doses of azo-proteins commensurate with adequate detection were employed to avoid saturation of the cells of one organ which would force into activity the cells of another organ not normally involved. Multiple doses were avoided for the same reason.

In this study, the interval of time between the injection of the azo-protein and the sacrifice of the animal was made as short as possible to reduce to a minimum the possibility of decomposition of the azo-protein *in vivo*. The coupling of a single azo-dye to several different proteins and the observation that differences do occur in the distribution of the azo-proteins thus formed excludes to a great degree the question of decomposition during the time intervals used. If the azo-protein were decomposed and the color group split from the protein molecule, a greater tendency for similarities rather than differences in distribution to occur should have been observed. Heidelberger, *et al.*(6), investigating the production of antibodies in rabbits against R-salt-azo-benzidine-azo-egg albumin, concluded that this dye-protein is not split *in vivo*.

It was observed in these experiments that when several different proteins were coupled to the same dye group, R-salt-azo-benzidine-diazonium, slight differences in the fate of the resulting azo-proteins when parenterally administered to mice were apparent. It was also observed that if a single protein, casein, is coupled to several different azo-compounds, differences in the distribution of the resulting azo-proteins again result. At the time that this paper was in preparation, Hawn and Janeway(7) clearly demonstrated that, when bovine serum albumin and bovine serum γ-globulin were administered intravenously to rabbits, lesions attributable to hypersensitivity were found in differing but characteristic locations for each protein. They concluded that the localization of bovine serum

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6. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1934, v59, 519.
7. Hawn, C. V. Z., and Janeway, C. A., *J. Exp. Med.*, 1947, v85, 571.

albumin differed from that of bovine serum γ-globulin. This is in accord with the differences in localization obtained when different proteins are coupled to a particular dye group.

It can easily be argued that the azo-proteins synthesized and used in this study were chemically heterogeneous. The available evidence indicates that this is so(8). Nevertheless, the conclusion that the localization of azo-proteins after parenteral injection may be modified by changes in either the protein or the dye component seems valid.

Summary. The distribution of a series of azo-proteins in mice after parenteral injection was studied by observing the intensity of color in the tissues on gross and microscopic examination. Their distribution appears to be modified either by the azo dye coupled to a particular protein or by the protein to which a particular azo dye is coupled.

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8. Gitlin, D., Latta, H., and Janeway, C. A., to be published.

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Development of Reticulum Cells and Lymphocytes in Transplants of Rabbit Lymph Node to Chick Chorioallantois.* (17835)

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The chorioallantoic membrane (CAM) of the developing chick embryo has proven to be a suitable host for various normal and malignant tissues derived from different species(1-4) and it was thought that grafting sections of lymphatic tissue from the rabbit onto the CAM of the developing chick embryo might be feasible. Cervical lymph nodes of guinea pigs were transplanted by Jaffe and Richter(5) into the abdominal wall of rats with regeneration of the lymphatic tissue and with apparent incorporation of the transplanted organ into the economy of the new host.

Technic. The popliteal lymph node of the rabbit was excised and bathed in buffered saline solution containing 300 units of penicillin and 60 μg of streptomycin per ml, then cut into thin slices and incubated in the saline solution at 37° C for ½ to 1 hour. The shell above the natural air sac of 9-day-old embryonated eggs was cut away, and the exposed shell membrane gently teased away from the CAM. A small amount of punctate bleeding of the CAM occurred, which was considered desirable, but eggs which bled profusely were discarded. A slice of lymphatic tissue was placed on the exposed CAM, directly over a surface blood vessel. The egg was then sealed with adequate amounts of Scotch tape and incubated at 37° C in the upright position. After suitable intervals the pieces of lymph node were cut away with the attached CAM and placed in 5% acetic acid Zenker's fixative. Hematoxylin- and eosin-stained paraffin sections were prepared for routine study. Some tissues were also fixed in Serra's solution and stained with methyl-green pyronine.

Results. Embryonated eggs containing slices of rabbit lymphatic tissue were incubated at 37° C for intervals of from 2 to

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* This study was supported by a grant from the Commonwealth Fund.

1. Goodpasture, E. W., Douglas, B., and Anderson, K., *J. Exp. Med.*, 1938, v68, 891.

2. Goodpasture, E. W., and Anderson, K., *Am. J. Path.*, 1942, v18, 563.

3. Blank, H., Coriell, L. L., and Scott, T. F. McNair, PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 341.

4. Kirber, H. P., Wiener Kirber, M., and Henle, W., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 481.

5. Jaffe, H. L., and Richter, M. N., *J. Exp. Med.*, 1928, v47, 977.

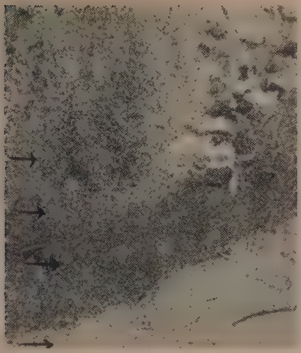

FIG. 1.
Explant of rabbit lymph node on chorioallantoic membrane (CAM) of chick embryo 4 days after implantation. 20 × H and E. Low power view showing from the top downward: two pieces of implanted lymph node; outgrowth of reticulum cells; area of vascularization from CAM (capillaries contain nucleated erythrocytes), with reticulum cells filling the spaces among the capillaries; edematous CAM.

10 days. After 2 days the nuclei of the lymphocytes, while easily discernible, were more dense and pyknotic than normal, and there was little tissue reaction. On the third day the lymphatic tissue was quite adherent to the CAM and the surrounding tissues were swollen. Histologically the CAM was edematous and the lymphocytes in the implanted tissue were undergoing degeneration. Between the implant and the membrane (Fig. 1) there was an area composed of capillaries full of nucleated erythrocytes extending from the CAM. Extending out from the implant toward and among these capillaries were typical reticulum cells. They were large and quite undifferentiated, with structureless eosinophilic cytoplasm and with large oval nuclei also optically empty except for small granules of basophilic material. These are well illustrated in Fig. 4. Where the reticulum cells occurred individualy, the cytoplasm could usually be seen trailing off to a point from each end of the wide oval nucleus. Much more frequently, however, the reticulum cells occurred in groups, without distinct borders of individual cells. On the fourth day the lymphocytes of the implant had undergone further degeneration. No cellular structure was in evidence, and rounded solid masses of material deeply stained with hematoxylin could be seen as the remains of nuclei of lymphocytes. The reticulum cells were present in far greater numbers. These occurred in broad bands set between the slice of lymph node and the CAM, and in many cases this band would expand into a broader mass of such cells beyond the point to which the implanted tissue extended. Many more capillaries filled with nucleated erythrocytes were seen in this area. In these capillaries leukocytes were quite rare. From the fourth to the tenth day not much change could be observed in the structures described, except for apparent further fragmentation of the residual material left from the nuclei of the implanted lymphocytes and deeper staining of the reticulum cells.

In a considerable number of specimens taken 4 or more days after implantation an additional change could be noted. Round or crescentic groups of fresh mature lymphocytes were seen at the periphery of the band of reticulum cells in the direction of the CAM, (Fig. 2) or set, as an island, within a mass of reticulum cells (Fig. 3 and 4). These groups of new lymphocytes never occurred in contiguity with the old, implanted lymphocytes, and in some cases were not near the capillaries from the CAM. The contrast between the appearance of the new and old lymphocytes, always with the band of reticulum cells interposed between them, was

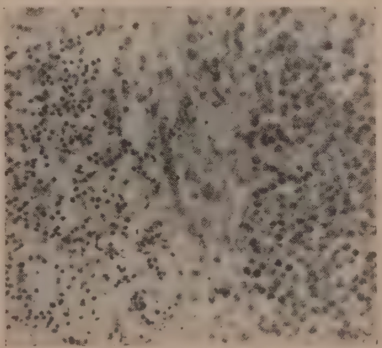

FIG. 2.
Similar preparation 5 days after implantation. 200 × H. and E. From left to right: debris of nuclei of disintegrating lymphocytes from implanted tissue; band of reticulum cells; new lymphocytes.

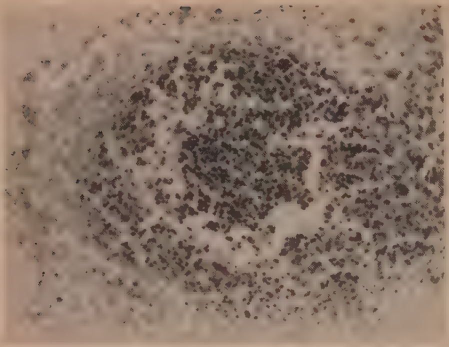

FIG. 3.
Similar preparation 6 days after implantation. 130 × H and E. This preparation shows a nest of new lymphocytes completely surrounded by reticulum cells, which have grown out to a considerable distance from the original implant.

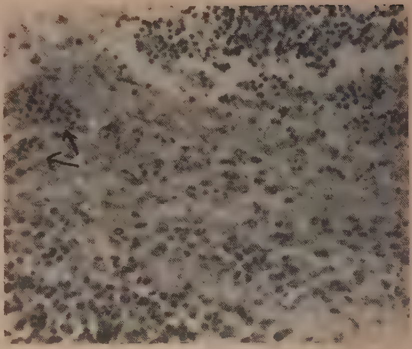

FIG. 5.
Similar preparation 5 days after implantation, showing especially the contrast between the degenerating nuclei of the implanted lymphocytes and the fresh nuclei of the new lymphocytes, with a band of reticulum cells between them. Arrows point to capillaries full of nucleated erythrocytes. 200 × H and E.

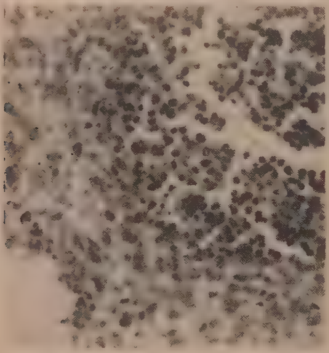

FIG. 4.
Portion of same field as that shown in Fig. 3, under higher power; showing in greater histologic detail the reticulum cells. The new lymphocytes and some transitional forms between them. 200 × H and E.

striking, as is seen in Fig. 5 and also Fig. 2. In many cases the cells at the border between adjacent areas of reticulum cells and new lymphocytes showed gradations between the two cell types with many fine degrees of difference in size and shape of nucleus and cytoplasm, and increasing basophilia and "hardening" of the nucleus. Such cells were not in general much larger than the mature lymphocytes.

All of the above observations were repeated in other experiments in which the tissues were fixed in Serra's solution and stained with hematoxylin and eosin. Other sections were cut from the latter blocks and stained with methyl green and pyronine, for desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) respectively. In these sections the reticulum cells took essentially no stain. The nuclei of the new lymphocytes were well stained with methyl green, indicating a considerabe degree of deposition of DNA in these cells, in contrast with the reticulum cells. As in the case of the other stain fine gradations in the amount of methyl-green stained nuclear material could be seen at the periphery of the groups of new lymphocytes. In contrast to these new cells, the remains of the nuclei of old lymphocytes were of a rather pale but distinct pink color in such sections. Other tissues were implanted from the rabbit onto the CAM of the chick for comparison with the observations made with the lymph node. In the case of rabbit spleen similar changes were observed to those described above. As control material, muscle, liver and kidney were similarly explanted. In each case there was outgrowth of various cell types, but none of these showed the development of reticulum cells or of lymphocytes. Of a total of over 100 graftings of lymphatic tissue undertaken thus far, more than 95% have been successful. Attempts to transplant explants to the CAM of other eggs have not thus far been successful.

Discussion. Several observations of those recorded above offer evidence of the successful grafting of the slices of rabbit lymph node on the CAM of the chick embryo. Among these is the intense vascularization of the area between CAM and implanted tissue, (with the nucleated erythrocytes in the capillaries indicating the origin of these blood vessels), and the position of the new reticulum cells and lymphocytes at the junction of CAM and implanted lymphatic tissue (indicating that elements from the explanted tissue were undergoing proliferation). The possibility was, of course, considered that the new lymphocytes found in later days might have emerged from the capillaries of chick blood, rather than have developed from the rabbit tissue, but several considerations seemed to make this possibility extremely remote. First, few leukocytes were seen within the capillaries of chick blood, and these were rarer outside the capillaries. Of those seen in the capillaries the great majority were neutrophils. Second, new lymphocytes were not found in all sections, although vascularization was in evidence in every case. Third, the distinct groups of lymphocytes mentioned above were not at all necessarily near capillaries but were always bordering on, or in immediate proximity to, the bands or groups of reticulum cells. In some cases, as shown in Fig. 3, the nest of lymphocytes was completely surrounded by reticulum cells. Finally, very subtle gradations in histologic form could often be seen by both stains between reticulum cells and lymphocytes at the junction of the two cell types. It would then appear to be entirely likely that these new lymphocytes were derived from the rabbit tissue, probably by some process of conversion from the reticulum cells which appeared first.

The chronologic appearance of reticulum cells and then, in close proximity, lymphocytes, is consistent with the findings of Jaffe and Richter on whole-organ transplants(5), and with results previously reported from this laboratory(6). In the latter study ribonucleic-acid-staining granules appeared in reticulum cells of the popliteal lymph node of rabbits 3 days after the injection of antigen into the foot pad. Five days after the injection of antigen these granules and crescents were seen in lymphocytes. The histologic characteristics of the ribonucleic-acid granule-containing cells changed in a manner which suggested that the lymphocytic hyperplasia known to occur in the lymph node after a local injection of antigen and during the period of antibody formation(6-8) was primarily a diffuse reticulum-cell hyperplasia, with gradual conversion of reticulum cells into lymphocytes. The observations made in the current study suggested in another way that the reticulum cells may be the immediate precursor of the lymphocyte, and that some observations which might be taken to imply growth of lymphocytes may in actuality imply multiplication of reticulum cells with subsequent conversion or maturation of these cells into lymphocytes.

Summary. Rabbit lymphatic tissue was placed on the CAM of the developing chick embryo and after incubation of 3 or more days evidences of successful grafting were obtained. Histologic sections of the tissues involved showed:

1. Appearance of many capillaries filled with nucleated erythrocytes.
2. Degeneration of the lymphocytes in the implanted tissue.
3. Appearance of bands of reticulum cells in a layer between the implanted lymph node and the CAM.
4. On occasion fresh mature lymphocytes appeared, always in proximity to the reticulum cells in the direction of the CAM. These cells occurred either in clusters at the periphery of the band of reticulum cells or in nests among the cells.

We are indebted to Dr. Philip R. Custer of the Presbyterian Hospital in Philadelphia for histologic consultations in the course of this work.

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Effect of Cold, Adrenocorticotropic and Thyroid Hormones on Urinary Excretion of Pentose in the Rat.* (17836)

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Green, Stoner and Bielschowsky(1) found that various forms of trauma are followed by an increase in the plasma concentration of total pentose and its phosphorylated fraction in the rabbit and the rat. These changes were of the same order as those produced by the intramuscular injection of fatal doses of adenosine triphosphate. McShan *et al.*(2) observed an increase in the pentose, inorganic phosphate, amino acid N, glucose, lactic and pyruvic acids of the blood of rats in shock induced by the Noble-Collip(3) and the tourniquet(4) methods. In studies of pentose metabolism in this laboratory the effects of various exogenous factors on urinary pentose excretion have been investigated. In this paper we are reporting the effect of cold, and of the administration of the adrenocorticotropic hormone (ACTH) and thyroid on urinary pentose excretion in the rat.

Rats of the Osborne-Mendel strain were placed on an adequate synthetic diet and water was allowed *ad libitum*. The urinary pentose in 24 hour samples of urine was determined by a modification of the method of Roe and Rice(5) for the determination of free pentose in animal tissues. To one part of urine was added 1 part of water, 4 parts of 5% Zn $SO_4 \cdot 7H_2O$ and 4 parts of 0.3 N $Ba(OH)_2$. The relatively large amounts of the Somogyi reagents used were necessary to obtain a satisfactory filtrate. The urine filtrate was treated in the same way as the filtrate of animal tissues in the Roe and Rice method.

Effect of cold. Twelve rats were placed in a constant temperature room at 31°C and control values were obtained on the urinary excretion of pentose over a period of 6 days. The animals were then placed in a constant temperature cold room at –1°C and the urinary pentose excretion was determined daily for a period of 6 days. A significant increase in the excretion of free pentose was observed ($P<0.01$). At the end of the 6 day period in the cold the animals were returned to the warm room at 31°C and the daily pentose excretion was determined for 6 days. In this follow-up period the urinary pentose excretion was essentially the same as observed during the original warm room period. These results are shown in Table I.

Influence of adrenocorticotropic hormone (ACTH) upon exposure to cold. Twelve rats were placed in a warm room at 31°C and control urinary pentose values were obtained for a 6 day period. The same animals were then transferred to a cold room at –1°C and 1 mg of ACTH[†] was given subcutaneously daily to each rat for 6 days. The daily urinary pentose excretion was determined during the latter period. As shown in Table I, the mean urinary excretion of pentose was slightly greater but this change is not statistically significant ($P =$ approximately 0.18). These data show that ACTH has an opposing influence upon the effect of cold on pentose excretion. The ACTH used produced glycosuria and increased uric acid ex-

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* Supported by a grant from the Division of Research Grants and Fellowships, the National Institutes of Health, United States Public Health Service.

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[†] Kindly supplied to us by the Armour Co.

TABLE I.
Effect of Cold and Administration of ACTH, Thyroid, and Thyroid Plus Thiouracil upon Urinary Pentose Excretion.
Data show average daily excretion of pentose as arabinose for periods of 6 days.

Experiment	No. of rats	Control 31°C Mean, mg	Control 31°C Range, mg	Experimental —1°C Mean, mg	Experimental —1°C Range, mg	Follow-up 31°C Mean, mg	Follow-up 31°C Range, mg
Effect of cold	12	2.47 ± .15	1.49-3.06	4.97 ± .19	4.01-6.15	2.54 ± .13	1.68-3.20
Effect of cold + ACTH	12	2.58 ± .12	1.91-3.57	2.93 ± .20	2.01-3.78	—	—
				Thyroid-fed 31°C		Thyroid + Thiouracil-fed 31°C	
Effect of thyroid	12	3.08 ± .25	1.25-4.42	6.20 ± .30	5.17-7.94	3.07 ± .16	2.01-3.99

The ± values are the standard error of the mean.

cretion in the rats and was thus shown to have biological potency.

Effect of thyroid. Twelve rats were placed in a warm room at 31°C and fed the basic diet, to which 2% of thyroid powder was added, for 6 days. As shown in Table I, the rats receiving thyroid powder showed a significant increase in the excretion of urinary pentose ($P<0.01$). The same animals were then fed the basic diet to which 2 g of thyroid powder and 2 g of thiouracil per 100 g of diet were added. The animals fed this diet showed a urinary pentose excretion practically the same as that observed during the control period.

Discussion. The method of Roe and Rice (5) does not determine appreciable amounts of combined pentose, as in this procedure the acetic acid reaction mixture is warmed at 70°C for only 10 minutes. The data reported are, therefore, for free pentose. It is possible that a part of the control values may be due to interfering substances. However, the increased values observed as a result of cold, or thyroid administration, very probably represent increases in true pentose. Evidence for this was obtained by the preparation of an absorption curve of the color produced with filtrate from a urine showing a high pentose value. The latter curve corresponded closely to the absorption curve of the color obtained with a pure arabinose solution.

The increased urinary pentose excretion resulting from cold appears to be due to increased metabolism mediated through the thyroid. Our results with thyroid feeding support this assumption. Under thyroid stimulation increased metabolism apparently brings about an increased cellular breakdown with an accelerated degradation of nucleotides. It has been shown that exposure to cold produces a rise in the metabolic rate and thyroid hyperplasia in the rat(6).

We observed a high concentration of free pentose in the blood of rats kept at –1°C. Blood studies will be reported later.

Summary. Low temperatures and thyroid administration increased significantly the urinary excretion of pentose in rats. ACTH administration had a preventive effect upon the increased pentosuria due to cold.

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Endogenous Creatinine in Serum and Urine.*† (17837)

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If the renal clearance of endogenous creatinine measured the rate of glomerular filtration, the infusion, injection or ingestion of a large quantity of material would be unnecessary in studies requiring an accurate estimation of glomerular filtration rate. The measurement of glomerular filtration rate with an endogenous substance would be of especial value in long term observations or in studies of fluid and electrolyte distribution and excretion. For this reason, we undertook to devise a method for measuring creatinine in plasma and urine.

Method. The method is essentially a modification of Borsook's(1), adapted for the use of small quantities of material available in studies on infants or small animals. Protein-free serum filtrate is prepared by pipetting 0.5 ml of serum and 1 ml of distilled water into a 13 x 100 mm tube, mixing with a glass rod, then adding 1 ml of 20% trichloroacetic acid with continuous stirring. The tube is stoppered and centrifuged in an angle centrifuge for 5 to 10 minutes. If the urine is protein-free, it is diluted with distilled water to a creatinine concentration between 0.1 and 0.5 mg%. When present, protein is precipitated with trichloroacetic acid.

Two ml of protein-free filtrate or diluted urine are pipetted into 13 x 100 mm tubes and 4 or 5 different aliquots of working standard, containing 0.25 or 0.50 mg% of creatinine in aqu. sol., are pipetted into similar tubes (usually 0.5, 1.0, 1.5 and 2.0 ml) and made up to approximately 2 ml with distilled water. Water (2 ml) serves as the blank. To each tube are added 0.2 ml of saturated oxalic acid and 15-20 mg of Lloyd's reagent. The tubes are stoppered and inverted or shaken occasionally for about 10 minutes. They are then centrifuged for about 5 min. in an angle centrifuge, and the clear supernatant aspirated off through a fine-tipped glass tube and discarded. Five parts of approximately 0.04 M picric acid solution are mixed with one part of 10% sodium hydroxide solution and diluted with 12 parts distilled water. This solution is made the day it is used. Into each tube are pipetted 2 ml of alkaline picrate solution, the original stopper is replaced, and the tube is well shaken to suspend the Lloyd's reagent. The tubes are allowed to stand for at least 10 minutes with occasional shaking and are then centrifuged in an angle centrifuge for about 5 minutes. Part of the supernatant from each tube is transferred with a Wintrobe pipette to a 10 mm cuvette and transmission is measured in a Beckman spectrophotometer (model DU) at 500 mμ against the blank set at 100% transmission. The colors are stable for at least 2 hours at room temperature.

Since blanks or standards diluted with trichloroacetic acid did not differ from the aqueous dilutions, no reagent blank for the serum filtrates was necessary. Plasma, either oxalated or heparinized, and serum showed no difference in creatinine concentration, and hemolysis does not seem to introduce an error. The only important source of error found was protein, which is adsorbed, eluted, and is a chromogen. Care must be taken, therefore, that the serum filtrate and the urine samples are completely protein-free. No chemical interference was found from high concentrations of urea, or from the presence of inulin, thiosulfate, ferrocyanide, or para-aminohippurate in the concentrations obtaining during renal function studies, although para-aminohippurate was found to give a direct Jaffé reaction.

Results and discussion. Recoveries of 0.5

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* This investigation was supported by a research grant from the National Heart Institute, United States Public Health Service.

† Presented before the American Physiological Society, *Fed. Proc.*, 1949, v8, 68.

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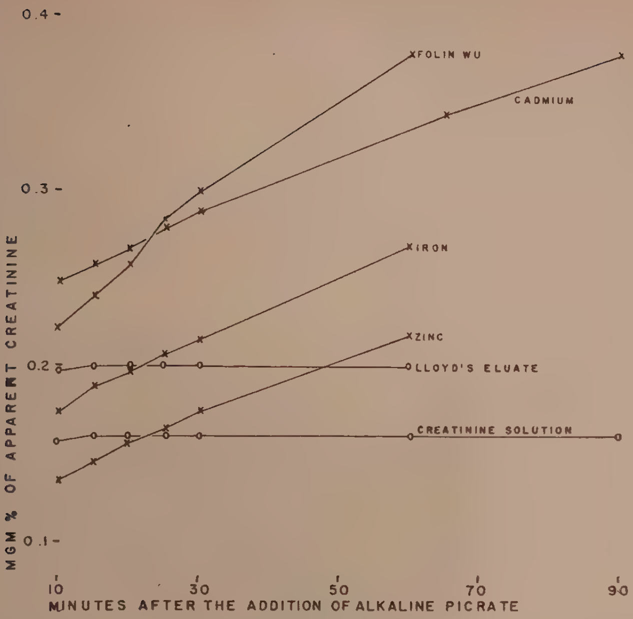

FIG. 1.

The increase in apparent creatinine concentration in Folin Wu, $CdSO_4$, iron, and Somogyi zinc filtrates of a single serum sample compared with the constant value of a Lloyd's eluate of the Folin Wu filtrate. All dilutions are 1:5. A creatinine solution is shown for comparison.

mg or more creatinine from pure solution or added to serum or urine are 98 to 102%. When 2 or more analyses of the same serum or urine are done, the maximum deviation from the average is ±1.5%. When serum and urine concentrations are increased by the administration of creatinine by mouth or vein to a level at which the error from non-creatinine chromogens is insignificant, creatinine determinations by this method agree with the direct Jaffé determination. And finally, when creatinine containing C^{14} was adsorbed from solution by this method, no C^{14} could be detected in the supernatant Lloyd's filtrate. The counts were made on dried preparations using a thin window Geiger-Muller tube(2). While the foregoing evidence shows that creatinine is quantitatively recovered by this method, it does not follow that other chromogens are not adsorbed from the plasma filtrate. The evidence on this point depends largely on observations first made by Hayman, *et al.*(3). They noticed that while creatinine solutions treated with alkaline picrate rapidly reached a constant color, plasma filtrates under the same circumstances continued to darken indefinitely, as would a mixed standard of creatinine and glucose. However, if the plasma filtrate or mixed standard were adsorbed on Lloyd's reagent and eluted, the chromogen in the eluate reached a constant color in a short time, as did a creatinine solution. We have repeated and confirmed this observation. Fig. 1 shows the increase in apparent creatinine concentration with time of 4 different protein-free filtrates of the same serum sample; the constant value of the Lloyd's eluate of the Folin Wu filtrate and of a creatinine solution are plotted for comparison.

When recovery of creatinine from the var-

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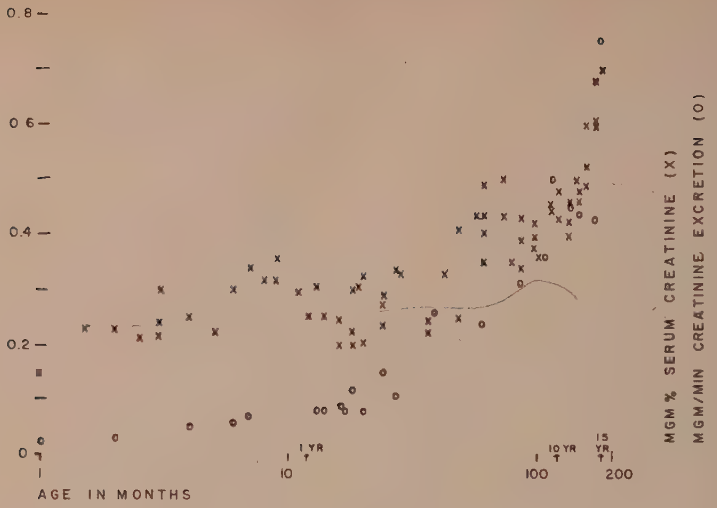

FIG. 2.
The relationship of age to serum creatinine concentration and to the urinary excretion of creatinine.

ious filtrates was compared, trichloroacetic acid, $CdSO_4$(4), and Folin Wu filtrates of the same sample showed the same creatinine concentration and gave 100% recovery of added creatinine; iron filtrates(5) gave concentrations and recoveries 10 to 18% lower. When the total chromogen concentration was considered to be the apparent creatinine concentration of a $CdSO_4$ or Folin Wu filtrate exactly twenty minutes after the addition of alkaline picrate, the normal sera studied showed that about 80% of this was creatinine. The quantity of chromogen recovered as creatinine from urine samples was 90 to 100%, and varied from one sample to another even in a single subject, although none of these urines contained measurable reducing material.

When the clearance of creatinine, as determined by this method, was compared with the inulin clearance in a series of 22 normal infants, children and adults(6), the average ratio of endogenous creatinine to inulin clearance was 1.03 and of exogenous creatinine to inulin clearance was 1.08.

The increase in serum creatinine concentration and in the rate of urinary excretion of creatinine with increasing age is shown in Fig. 2. These data were collected in order to establish the expected values for various ages. Age, rather than weight was selected as the criterion since children with severe malnutrition or obese subjects of similar age and development had closely comparable serum concentrations. However, in children whose growth was severely retarded or precocious, serum levels agreed more closely with apparent than with chronological age. Probably surface area would be the best criterion, if more reliable estimates were available for infants and children. In our experience only subjects with impaired renal function have serum concentrations significantly higher than normal, and we consider an elevated serum creatinine as evidence of such impairment. Three cases of amyotonia congenita are the only subjects in our experience whose serum levels were significantly low, on the basis of either apparent or chronological age. Only a few observations have been made on full-term infants below the age of

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one month; they indicate that creatinine at birth is at or near the adult level and falls within about two weeks to the level of the month old infant. Premature infants show serum levels which are higher and rates of excretion which are lower than full term infants of the came postnatal age; this is undoubtedly related to the degree of immaturity of the kidney in these infants.

Conclusions. (1) The technic described measures all of the creatinine and only creatinine in blood and urine. (2) There is a gradual rise in the serum concentration and in the rate of urinary excretion of creatinine from one month of age to puberty, when adult levels are reached.

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Dicumarol Labelled with C^{14}.* (17838)

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As a result of the classical work of Link(1), dicumarol has come into widespread clinical use as a therapeutic agent against thrombosis. However, there has been little information available regarding the mechanism whereby dicumarol lowers the prothrombin level in the blood until the recent demonstration by Lupton(2) that after treatment of rats with dicumarol, the livers fail to add prothrombin to blood perfused through them. In an attempt to obtain further information regarding its action, dicumarol labelled with radioactive C^{14} was used in animal experiments.

Methods. Dicumarol, labelled with C^{14} in the methylene bridge, was synthesized using formaldehyde-C^{14}. The labelled dicumarol was dissolved in 0.1 N sodium hydroxide (5 mg per cc) and injected intravenously in mice and rabbits. 0.05 cc of solution (0.25 mg) was given to each mouse, while each rabbit (weight 2 kg) received 2.0 cc. To determine radioactivity in the blood, samples were taken, weighed, dried and the material spread uniformly in a platinum dish. Other tissues were treated similarly. Radioactivity measurements were then made with the end-window type of Geiger-Muller chamber with a scale of 128 counting unit. Correction for self-absorption of C^{14} activity was made. Prothrombin times were determined by the usual methods using 0.02 M calcium chloride solution.

Results. Fourteen mice were each given 0.25 mg of labelled dicumarol of specific activity 120.4 registers/min./mg intravenously (1 register = 128 counts). The first 4 mice were placed in a metabolism apparatus and the expired CO_2 collected and tested for radioactivity. No radioactivity whatsoever could be determined in the expired air and as a consequence, it was not necessary to collect expired air in further experiments. At various times after the injection, the animals were sacrificed and after weighing, the radioactivity determined for lungs, blood, liver, gall-bladder, kidneys, stomach, intestines, intestinal contents, feces and urine. No significant amount of activity could be detected in the lungs. Results for other tissues are shown in Fig. 1. Large amounts of radioactivity were detectable in the liver, intestines, gall-bladder, feces and urine. A trace was found in the kidney and stomach. Activity was determined separately on the intestine and in-

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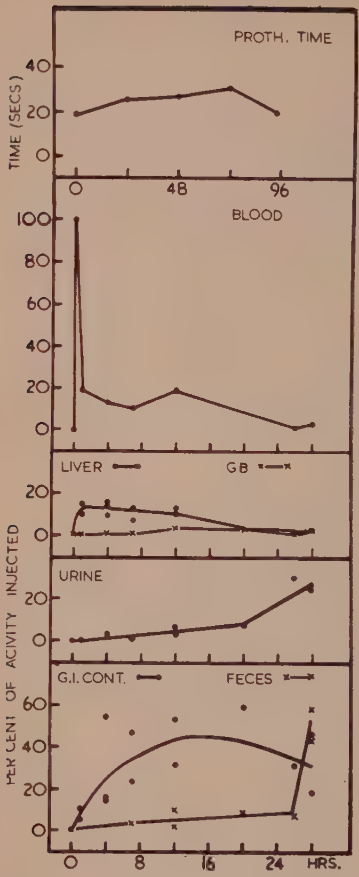

FIG. 1.

Blood and tissue levels of radioactivity in mice following C^{14}-labeled dicumarol. 0.25 mg (= 10 mg/kg) injected intravenously at zero time. Value for urine and feces is total excretion to time shown. G.I. Cont. = Gastro-Intestinal Contents. G.B. = Gall Bladder and Contents.

testinal contents and feces. Since it was found that the value for the intestine was the lower, the more effective the removal of intestinal contents, values for intestine and intestinal contents have been reported together while the true values for the intestine proper are probably quite small. The kidneys contained about 2-4% of the activity until 12 hours following injection. After 18 hours only a trace of activity remained in the kidney. The specific activity was at no time greater than that of the lung and hence the activity found was due to the blood present in the kidney. It was of interest to find that the bile showed the highest specific activity per gram of wet tissue, one sample of bile showing twice the initial concentration of radioactivity in the blood. It is to be noted that the values given in Fig. 1 for tissues and intestinal contents show actual values for activity at the given time, whereas urine and feces show total excretion from zero to the given time. Values for urine include the radioactivity found in the urinary bladder. Determinations of the activity of plasma from the blood samples demonstrated that all the activity was in the plasma, none in the cells.

Fig. 1 shows very interesting features in the time relationships of the appearance of the activity in different organs. The activity disappeared rapidly from the blood. After one hour, the total activity was distributed between blood, liver and intestine in decreasing order. From the 1st to the 12th hour, levels of activity in blood and liver remained approximately constant but that in the intestinal contents increased until it represented the major part of the activity. After the 12th hour, the activity in liver and blood fell to a low level. However, appreciable activity was now found in the urine and this increased until by the 28th hour, 30% of the dose had been excreted in the urine. At the termination of the experiment at the 28th hour, a high percentage of the activity had appeared in the feces. Judged by the radioactivity, these results indicate that dicumarol disappeared rapidly from the blood and was taken up by the liver. One hour after the injection, 10-15% of the injected dicumarol was present in the liver. At this time there was very little activity in other tissues. However, while there was little decrease in the level of dicumarol in the liver after four hours, a large amount of the activity was now found in the intestinal contents. The very high specific activity found in the bile suggested that this activity reached the intestine from the liver via the bile ducts. After the fourth hour, activity began to appear in the kidneys and urine, although urinary excretion of activity was not significant until after 12 hours and was greatest between the 18th and the 28th hour. This suggested that

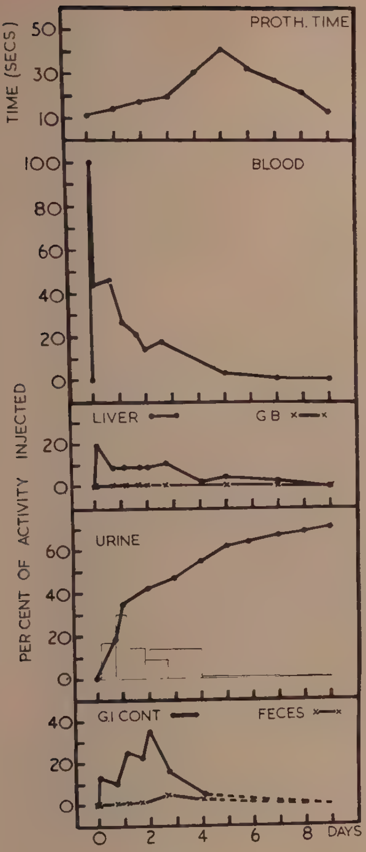

FIG. 2.

Blood and tissue levels of radioactivity in rabbits following C^{14}-labeled dicumarol. 5 mg/kg injected intravenously. For urine, histograms show 24-hour excretion prior to death; continuous line shows total excretion for a single rabbit followed for the whole experiment.

urinary excretion was not due to the injected dicumarol but to activity that had been reabsorbed from the intestinal tract. This was probably also responsible for activity observed in the blood after the 7th hour. At the 28th hour the activity in tissues was practically nil as the result of excretion, chiefly in the feces, although 30% of the activity was found in the urine.

The results for a series of rabbits, similarly injected with radioactive dicumarol, are shown in Fig. 2. Again there was a rapid, though slower, disappearance of dicumarol from the blood. In 4 hours, 30% of the injected dicumarol was found in the blood. The activity of the liver rose rapidly to 20% of the injected activity after 2 hours and then fell to about 10% and remained there for 3 days, at which time the prothrombin time started to rise and radioactivity in the liver fell to about 2% of the dose. The intestinal contents again showed activity, about 12% of the activity being found in them 2 hours after the injection and reaching 25% of the dose after 24 hours. However, in contrast to the mouse, there was practically no significant activity found in the feces even at the termination of the experiment, when the total amount of activity excreted in the feces was less than 5%. Activity was found in the urine. Excretion of 20% of the activity was found in the urine and urinary bladder by the 18th hour and approximately 72% of the total activity was recovered from the urine by the end of the experiment. The kidney showed no higher specific activity than the lung, etc. and the amount in the kidney at no time exceeded 6% of the dose injected. Again, there was considerable activity found in the bile and gall-bladder, this material having the highest specific activity of any tissue. These results were similar to those obtained in the mouse with the exception that no excretion of the activity was found in the feces. Apparently all the activity excreted into the intestine by the liver via the bile was reabsorbed and excreted by the kidneys. It can be observed that the activity remained in the liver much longer for the rabbits than for the mice.

Nature of the activity in the liver. In order to determine whether the radioactivity observed in the liver was due to C^{14} still attached to dicumarol, a rabbit was injected with 10 mg of labelled dicumarol (specific activity, 140 registers/min/mg) and after 24 hours was sacrificed and the liver removed. The liver was comminuted and to it was added 29 mg of inactive dicumarol to provide a carrier. After mixing thoroughly, the activity was extracted from the liver with acetone. Most of the acetone was removed by distillation, sodium hydroxide was added and the mixture extracted three times with ether. The alkaline solution was now acidified with

HCl and the dicumarol extracted with ether. On evaporation of the ether, a crude product was obtained containing all the radioactivity of the original liver. All fractions discarded were checked for radioactivity and no significant activity could be detected in these fractions. The crude dicumarol so obtained was subjected to repeated recrystallizations from cyclohexanone. After 5 recrystallizations, 2.6 mg of product was obtained with a melting point of 288°C, mixed melting point of 288°C, and a specific activity of 4.1 registers/min/mg. If the number of mgs of labelled dicumarol present in the liver (before adding carrier) is X; $(X + 29)\,4.1 = X \times 140$ and $X = 0.88$ mg *i.e.* 8.8% of the injected dicumarol. The total activity in the liver before extraction was 120 registers per min representing 8.6% of the activity injected. The close agreement between these two figures is direct evidence that the activity found in the liver, following the injection of radioactive dicumarol is essentially all in the form of unchanged dicumarol. This means that for the liver, the activity gives us a reasonably accurate measure of the dicumarol present.

In a further test, dicumarol isolated from liver (6.1 mg, specific activity 4.1 reg/min/mg) was converted to the diacetate (M.P. 250-252°C, mixed M.P. 250-252°C). Specific activity of the diacetate was 3.3 (found) and 3.3 (calc) regs/min/mg.

Dicumarol and vitamin K. It has been shown by a number of workers(1), that simultaneous administration of dicumarol and vitamin K reduces the action of dicumarol. A series of mice were therefore injected with 0.25 mg dicumarol together with 1 mg of vitamin K (2-methyl-1, 4-naphthoquinone phosphoric acid ester, Synkavite). It was found that excepting for the liver, there was no significant difference in the activity found in tissues from these animals, compared to mice receiving radioactive dicumarol without vitamin K at the same time. The activity found in the liver is shown in Fig. 3. It can be seen that the activity twenty minutes and one hour after the injection was the same for the mice which received vitamin K as for those which did not. However, after the first hour the animals receiving vitamin K showed much less activity in the liver suggesting a more rapid displacement of dicumarol from the liver in the presence of vitamin K. Some dicumarol remained in the liver after 18 hours, at which time the values were about the same as for the animals without vitamin K.

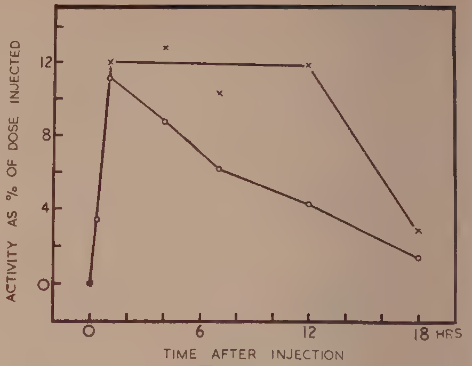

FIG. 3.

Dicumarol in mouse liver. x———x after intravenous injection of 0.25 mg C^{14}-dicumarol; o———o after 0.25 mg of C^{14}-dicumarol + 1.0 mg of vitamin K (2-methyl-1,4-naphthoquinone phosphoric acid ester).

Discussion. It is evident from these results that on administration, dicumarol was carried to the liver and remained fixed there for a considerable period. This period varied with the animal species and presumably this may be the cause of variations in the effectiveness of prothrombopenic agents between different animal species. Thus in the mice (Fig. 1) the prothrombin time reached its peak value on the third day and returned to normal values on the fourth day. In the rabbits (Fig. 2), the prothrombin time reached its peak value (38-42″) on the third day but this value was maintained for 2 days and the prothrombin time did not reach normal values until the ninth day. It is significant that dicumarol remained in the mouse liver only 16 hours, and none could be detected after 24 hours, while the level of dicumarol in the rabbit liver remained constant (about 1 mg) for 3 days after the injection and a small amount was still present on the fifth and seventh days. This indicates a direct relationship between the duration of fixation of dicumarol in the liver and the duration of the prothrombinopenia in the blood. Vitamin K appeared to interfere with the fixation of dicumarol in the liver in such a way that the dicumarol was displaced from the organ more quickly.

Since no radioactivity appeared in the urine for some hours after intravenous injection of labelled dicumarol, it is evident that in mice and rabbits, the dicumarol was not excreted by the kidney. This has also been reported by Weiner, Axelrod, Shapiro and Brodie(3) in man. However, the activity appeared in the bile and gastro-intestinal tract shortly after administration and much later it was found in the urine, indicating metabolism of the dicumarol. In the rabbit, all of the labelled groups in the dicumarol was reabsorbed and excreted in the urine but in the mouse, a part of it appeared in the feces. The nature of the product excreted is now under investigation. Thus far, it has been shown that the activity in the urine is not dicumarol, agreeing with the suggestion above that the dicumarol has been metabolised. In order to ensure complete absorption, the dicumarol used was administered intravenously. However, experiments have been conducted with the material given orally with essentially the same results.

Summary. Dicumarol, containing C^{14} in the methylene bridge was synthesized and given intravenously to mice and rabbits. The activity disappeared rapidly from the blood and was recovered in the liver, bile, intestinal contents and later in the urine, but not from other tissues. About 10% of the activity became fixed in the liver. It was demonstrated by isolation using the isotope dilution technique that this activity was due to unchanged dicumarol. This dicumarol remained fixed in the mouse liver for 16 hours, in rabbit liver for 3 days. The corresponding duration of hypoprothrombinemia was 4 days and 9 days. The presence of extra vitamin K resulted in the dicumarol disappearing more rapidly from the liver. It is suggested that the period of time that dicumarol remains in the liver is related to the effectiveness of the agent in interfering with the formation of prothrombin. Dicumarol was not excreted by the kidney but evidence of excretion of metabolic products was found in the urine and feces for the mouse and in the urine only for the rabbit.

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Relation of Adrenal Cortical Steroids to Antibody Release. (17839)

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That the lymphocyte is concerned with the manufacture and storage of antibodies has been demonstrated by a number of investigators(1,2,3,4,5). The administration to normal animals of those adrenal cortical steroids oxygenated at C_{11} or adrenotrophic hormone produces involution of the thymus, degeneration of lymphocytes in lymphoid tissue, a peripheral leucopenia accompanied by a granulocytosis and a decrease of lymphocytes in thoracic duct lymph(6,7,8,9,10,11,12,13). The lymphocyte has been shown to contain normal serum gamma globulin(14,15) the release of which is under adrenotrophic control(15). Furthermore, it has been demonstrated that adrenal cortical hormones or adrenotrophic hormone administered to an immune or hyperimmune animal produces a marked rise in antibody titer(16,17,18). The anamnestic reaction has been produced in intact animals by means of adrenal cortical steroids or adrenotrophin(19). Whether or not adrenal cortical hormones will induce a disintegration of circulating lymphocytes is not known(9,20). The present investigation was undertaken in an attempt to answer this question.

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* This work was submitted in thesis form by the senior author as a partial requirement for the degree of Doctor of Philosophy at the University of Illinois Graduate College, Chicago, Ill.

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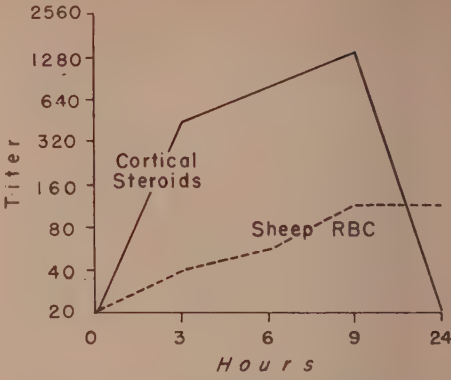

FIG. 1.

Anamnestic response induced by means of the antigen and adrenal cortical steroids in oil.

The initial step was the reproduction of the work reported by Dougherty(19) in regard to inducing the anamnestic response by means of adrenal cortical steroids. Eight albino rabbits of both sexes weighing between 4-5 lb. were immunized against sheep erythrocytes in exactly the fashion described by Dougherty *et al.*(19). Hemolysin and agglutinin titers were determined on each animal before and at intervals during the period of immunization. The average peak titer obtained after 2½ months was 1:3200. The animals were then kept until their titers fell to approximately the initial levels. At that time 4 of them were given a "booster" dose of antigen consisting of 2 cc of a 5% suspension of sheep red blood cells administered intravenously. Each of the remaining 4 was given subcutaneously into the skin of the back 5 cc of Upjohn's Lipo-Adrenal Cortex. Blood samples were obtained from individual rabbits by slitting the marginal ear vein with a razor blade and collecting the blood in a small test tube. Samples were secured at 3, 6, 9 and 24 hours after the administration of the antigen or the cortical steroids, and hemolysin determinations were made. The results are recorded in Fig. 1. The peak titer following the injection of cortical steroids was not as high as that reported by Dougherty *et al.*(19) and it was reached at 9 hours rather than 6 hours. It was definitely much higher, however, than that secured with the antigen. Whereas it had fallen to its initial level within 24 hours, the titer obtained after the "booster" dose of antigen was still at its peak at 24 hours. The work of Dougherty, Chase and White(19) having been confirmed, the body of the experiment was undertaken.

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TABLE I.
Normal Rabbits Receiving 4 cc of "Immune" Lymphocytes.

Rabbit No.	Wt, lb	Lymphocytes received			
		No./mm³ (in 1000's)	Total No.	No./cc rabbit bld.	% lymphs in suspension
C5	4	500	200×10^7	2083×10^4	99
C6	4½	650	260×10^7	2708×10^4	98
C7	4	1,090	436×10^7	4541×10^4	96
C8	4	920	368×10^7	3833×10^4	95
Normal Rabbits Receiving 4 cc of "Immune" Lymphocytes and 5 cc of Cortical Steroids.					
C9	4½	550	220×10^7	2291×10^4	98
C10	4	420	168×10^7	1750×10^4	99
C11	4	720	288×10^7	3000×10^4	96
C12*	4	530	212×10^7	2208×10^4	97

* Died after cells inj.

The number of cells per cc of rabbit blood calculated by determining the blood vol. of each rabbit (48 cc/kg body wt) and dividing that into the total number of cells received.

All animals used in the experiment were female albino rabbits weighing between 4-5 lb. Hemolysin tests were made on each rabbit previous to immunization, periodically during immunization and as indicated on recipient rabbits. Lederle amboceptor was employed and the complement was either Lyovac or was secured by bleeding 3 guinea pigs from the heart and pooling the sera. Amboceptor and complement were titrated in the usual manner prior to each hemolysin test done. Sixteen rabbits were injected intravenously on alternate days with 2 cc of a 5% suspension of fresh, washed sheep red blood cells for a 2½ month period. At the end of this time the animals were killed in pairs by anesthetizing them lightly with ether and then exsanguinating them by heart puncture. The thymus, large mesenteric lymph node and appendix were removed from each rabbit. The appendix was slit and thoroughly washed free of fecal material with warm saline. All tissues were rinsed 3 times in separate beakers of physiologic salt solution standing in a 37°C water bath. They were then placed in 50-75 cc of warm saline and were minced as finely as possible with a scissors. The milky supernatant saline was strained through 2 layers of surgical gauze to remove the gross pieces of tissue and the cells were washed 3 times in warm salt solution by centrifugation at 1500 r.p.m. for 10 minutes. A sample of the third washing was saved for hemolysin determination as a check that all free hemolysin had been removed. Between each washing, after the saline had been added and the cells resuspended, the mixture was stirred slowly with wooden applicators to remove fibrin. This procedure continued until no further fibrin collected around the applicators. After the last washing the packed cells were suspended in 5 cc of warm saline. A count of the cells contained in this amount was done by means of a red blood cell counting pipette, and a smear was made and stained with Giemsa to determine the percentage of lymphocytes present (Tables I and II). One cubic centimeter was then removed and placed in a separate test tube. These cells were frozen and thawed 3 times to extract antibody from the lymphocytes. Following the third thawing the material was centrifuged slowly at 1500 r.p.m. for 5 minutes to remove cellular debris and hemolysin tests were made on the supernatant fluid to determine the amount of antibody present per cubic centimeter of cellular suspension injected. The remaining 4 cc of lymphocytic suspension were injected very slowly intravenously into a normal rabbit as soon as possible after recovery from the original animals. Leucocyte and differential counts were made 15, 30 minutes, 1, 3, 6, 9 and 24 hours following the injection of cells, and samples of blood for hemolysin determinations were secured at 3, 6, 9 and 24 hours following the administration of the cells. Of the 16 immune animals, 8 donated cells to 4 normal rabbits which received only the cells, and 8 donated cells to 4 normal rabbits each of

TABLE II.
Immunized Rabbits Receiving "Immune" Lymphocytes.
Immune Controls.

			Lymphocytes received			
Rabbit No.	Wt, lb	No. cc	No./mm³ (in 1000's)	Total No.	No./cc rabbit bld	% lymphs in suspension
C20*	4	4½	240	100×10^7	1050×10^4	97
C21*	4	10	145	145×10^7	1510×10^4	96
C1	5½	4	540	216×10^7	1800×10^4	97
C2(H)	5½	4	900	360×10^7	3000×10^4	98
C3*	5	4	810	324×10^7	2700×10^4	95
C4*	5	4				96
Immunized Rabbits Receiving "Immune" Lymphocytes and 5 cc of Cortical Steroids.						
C13	5½	4	790	316×10^7	2550×10^4	95
C14(H)	4	4	760	304×10^7	3166×10^4	96
C15	5½	5	390	195×10^7	1625×10^4	99
C16*	4	4	700	280×10^7	2916×10^4	97
C17	4	6	470	282×10^7	2937×10^4	98
C18	4	3½	84.6	29×10^7	308×10^4	98
C19	4	4½	330	148×10^7	1546×10^4	96

* Died after cells injected.
(H) Received heparin.

TABLE III.
Hemolysin Titers in Normal Animals Receiving "Immune" Lymphocytes and Cortical Steroids.

Rabbit No.	Initial	3 hr	6 hr	9 hr	24 hr	96 hr	6 days	14 days	Lymph ext.	3rd washing	Donor rabbits
C13	0	0	1:20	1:40	1:100		1:100	1:40	1:1600	0	No. 83 1:3200 No. 100 1:1600
C19	0	1:20	1:20	1:20	1:20				1:1280	0	No. 17 1:1280
C14(H)	0	0	0	0	0	0			1:3200	0	No. 94 1:1600 No. 95 1:6400
C15	1:40	1:40	1:40	1:40	1:40				1:1600	0	No. 77 1:800 No. 86 1:3200
C17	1:20	1:20	1:20	1:20	1:20						No. 9 1:800
C18	0	0	0	0	0				1:640		No. 21 1:2560
C16*	0									0	No. 81 1:3200 No. 84 1:3200
C15′*	0								1:1600	0	No. 77 1:800 No. 86 1:3200

* Died after cells inj. (H) Received heparin. C15′ received 1 cc lymphocytic suspension and died. The remaining 3½ cc diluted to 7 cc and C15 received 5 cc of this.

which was given subcutaneously into the skin of the back 5 cc of Lipo-Adrenal Cortex 30 minutes before the cells were injected.

In addition, 2 control groups of normal animals were done. Each of 4 normal rabbits received intravenously lymphocytes secured in the aforementioned manner from 2 normal rabbits. Each of 4 additional rabbits received intravenously lymphocytes secured from 2 normal rabbits and 5 cc of Lipo-Adrenal Cortex injected subcutaneously 30 minutes before the cells. Leucocyte counts and differentials were done on these animals and blood samples were obtained at the same times as for the test rabbits.

As can be observed in Table III animals C17, C18 and C19 each received lymphocytes secured from one immune rabbit. These animals were used as a preliminary experiment. Thereafter cells from 2 animals were pooled in order to insure having a sufficient number of lymphocytes.

The fate of the circulating lymphocyte is unknown. There are, however, several theories as to their disposition, one of which is that they pass through the mucosa of the gastrointestinal canal and are lost(21). Erf (22) has shown that this is probably not the case. When gastro-enterectomized rabbits were injected intravenously with autogenetic or homogenetic lymphocytes an immediate leucopenia accompanied by a lymphocytosis and granulopenia which persisted for 30 minutes was observed. At the end of this time there occurred a leucocytosis associated with a granulocytosis and a lymphopenia which was observed for 6 hours. Erf concluded the injected lymphocytes circulated for 30 minutes.

Faludi(23), however, after administering polymorphonuclear leucocytes intravenously to rabbits also observed an immediate leucopenia accompanied by a lymphocytosis and a granulopenia which persisted for 30 minutes. Later the leucocytosis, granulocytosis and lymphopenia occurred as described by Erf (22).

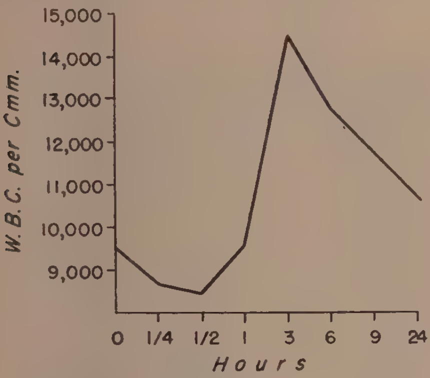

FIG. 2.
Leucocyte count following the intravenous injections of lymphocytes. Average of 4 animals.

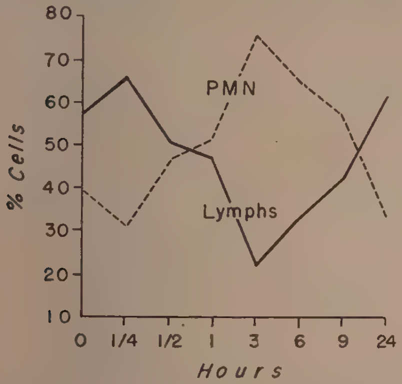

FIG. 3.
Polymorphonuclear and lymphocyte percentages following the intravenous injection of lymphocytes. Average of 4 animals.

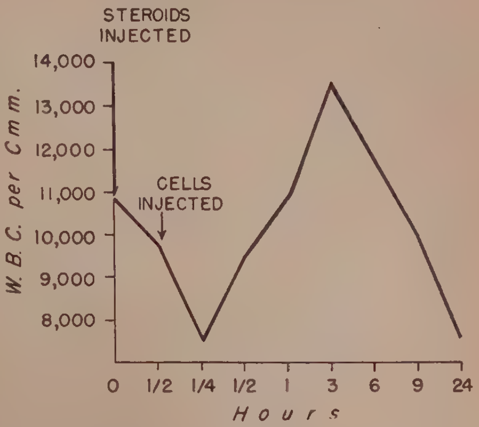

FIG. 4.
Leucocyte count following the injection of adrenal cortical steroids in oil and lymphocytes. Average of 3 animals.

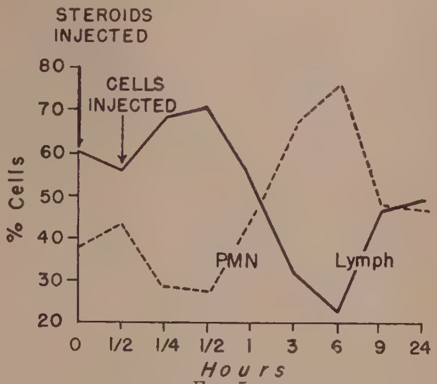

FIG. 5.
Polymorphonuclear and lymphocyte percentages following the injection of adrenal cortical steroids in oil and lymphocytes. Average of 3 animals.

Both of these reactions might be explained on the basis of parenterally administered protein which has been shown to produce a lymphocytosis(24,25).

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21. Drinker, C. K., and Yoffey, J. M., *Lymphatics, Lymph, and Lymphoid Tissue*, Harvard University Press, Cambridge, Mass., 1941.
22. Erf, L. A., *Am. J. Med. Sci.*, 1940, v200. 1.
23. Faludi, F., *Folia Hematol.*, 1938, v59, 357.
24. Rich, A. R., Lewis, M. R., and Wintrobe, M. M., *Bull. Johns Hopkins Hosp.*, 1939, v65, 311.
25. Wiseman, B. K., *J. Exp. Med.*, 1931, v53, 499.

TABLE IV.
Hemolysin Titers in Normal Animals Receiving "Immune" Lymphocytes.

Rabbit No.	Initial	3 hr	6 hr	9 hr	24 hr	Lymph ext.	3rd washing	Donor rabbits	
C20*	0							No. 18	1:3200
C21*	0							No. 19	1:3200
C1	0	0	0	0	0	1:3200	0	No. 80 No. 87	1:1600 1:6400
C2(H)	0	0	0	0	0	1:3200	0	No. 76 No. 91	1:3200 1:6400
C3*	0					1:3200	0	No. 92 No. 93	1:6400 1:3200
C4*	0					1:1600	0	No. 88 No. 89	1:1600 1:3200

* Died after cells inj.
(H) Received heparin.

It became necessary, therefore, to establish how long the injected lymphocytes under consideration circulated. Figs. 2 and 3 are averages of blood counts observed in 4 rabbits each receiving lymphocytes pooled from 2 normal rabbits. As can be seen, the observations of Erf(22) and Faludi(23) are confirmed. Figs. 4 and 5 are averages of 3 rabbits which received Lipo-Adrenal Cortex 30 minutes before lymphocytes pooled from 2 normal rabbits were injected. Thirty minutes after the administration of cortical steroids the leucocyte count fell and simultaneously there occurred a lymphopenia with agranulocytosis. This is in agreement with previous reports(7,8,9,10,11,13). Upon injecting the cells the leucopenia became more pronounced but the lymphopenia and granulocytosis were replaced by a lymphocytosis and a granulopenia which persisted for 30 minutes.

No antibody could be detected in the sera of the 8 normal rabbits receiving intravenously lymphocytes from normal rabbits. The administration of cortical steroids did not alter these results.

In Tables III and IV are recorded the results of hemolysin titer determinations made on both groups of animals receiving "immune" lymphocytes. Two animals which received cells and cortical steroids were positive, one very definitely. That this was a true transfer of antibody is indicated by the fact that it persisted so long. Previous workers(26,27) who transferred antibody by means of emulsifying spleen from an immune animal and injecting the cells intraperitoneally into a normal animal reported being able to detect antibody in the normal animal for as long as 30 days.

In spite of the 2 positive rabbits, it is concluded that under the conditions of the experimental method adrenal cortical steroids do not cause circulating lymphocytes to disintegrate and release antibody. The peak titer in the definitely positive animal (C13) did not come until 24 hours after the cells were injected. The effect of a single dose of cortical steroids is completely expended in 24 hours(17,18). Furthermore, the injected lymphocytes do not circulate that long. Since the cortical steroids did not have to penetrate lymphoid tissue to produce their effect in this instance but simply needed to be in the blood stream, the peak should have come much earlier. The steroids were injected 30 minutes before the lymphocytes. That a sufficient amount had been absorbed to affect lymphocytes in lymphoid tissue is indicated by the leucopenia and lymphopenia which were detectable in 30 minutes. Therefore, adequate amounts should have been in the circulating blood stream to act on the injected cells. These cells circulated only 30 minutes while the cortical steroids depressed the animal's own lymphocytes. Consequently, the lymphocytosis which occurred for 30 minutes after the injection of cells was proba-

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26. Luckhart, A. B., and Becht, F. C., *Am. J. Physiol.*, 1911, v28, 257.

27. Topley, W. W. C., *J. Path. Bact.*, 1930, v33, 339.

bly due to the injected cells.

A striking phenomenon was encountered during the course of the experiment. Of 8 animals receiving cells from normal rabbits, 1 died following the administration of cells or 12%. As can be observed from Tables III and IV of 13 animals receiving lymphocytes from immune rabbits, 6 died or 45%. The deaths were sudden and occurred 1-2 minutes after the cells were injected. The animal's ears became cold, it developed cyanosis, marked respiratory distress, and convulsions followed by death. The heart beat after the animal ceased to breathe but artificial respiration or adrenalin given either intravenously or intracardially was of no avail. The deaths were not due to embolism apparently. The injected cells were evenly dispersed and were not agglutinated by the recipient rabbit's serum. Fibrin was known to be present. Thromboplastin was present in large amounts due to the use of the thymus as a source of cells. That should have been removed during the washing process, however. Nevertheless, 2 animals were heparinized before the cells were injected and these rabbits survived. One cc of Abbott's Sodium Heparin containing 10 mg of purified sodium heparin standardized to contain 1000 provisional International Units was injected intravenously 5 minutes before the cells. According to Jorpes (28) heparin neutralizes complement and may be concerned in the immunologic process. It was felt that a variable of unknown quantity was being introduced and the use of heparin was discontinued.

It became evident that the greater percentage of deaths followed the injection of "immune" cells and might be due to shock of the anaphylactic type. None of the animals showed right heart dilatation, however, and no gross pathology was found except in one animal whose intestinal and mesenteric vessels appeared engorged. This animal showed clots in both chambers of the right heart also. Histologic examination of tissues from one animal were made. The only pathologic finding was constriction of the pulmonary arteries. This finding plus the type of death could very well indicate shock.

An explanation of shock on the basis of Forssman antigen was considered but the rabbit has no Forssman antigen. According to Landsteiner(29), the occurrence of immunologically similar substances in the cells of unrelated species is not limited to Forssman antigen. The presence of such an antigen in the rabbit and its antibody in the "immune" lymphocytes would explain a shock reaction. This problem should be investigated further.

Conclusions. 1. The anamnestic reaction was produced by means of subcutaneously administered adrenal cortical steroids in oil to rabbits immunized against sheep erythrocytes.

2. Lymphocytes obtained from the appendix, thymus and mesenteric node of rabbits when injected intravenously into a normal rabbit circulate for a period of about 30 minutes only.

3. Lymphocytes obtained from the appendix, thymus and mesenteric node of rabbits immunized against sheep erythrocytes when injected intravenously into a normal rabbit do not release antibody under the conditions of the experimental method employed.

4. Adrenal cortical steroids administered to a normal rabbit which also receives lymphocytes intravenously from rabbits immunized against sheep erythrocytes do not induce a release of antibody from these cells while they are circulating according to the experimental method employed.

The authors are indebted to the Upjohn Co., Kalamazoo, Mich., for 40 cc of the 80 cc of Lipo-Adrenal Cortex used in the experiment.

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28. Jorpes, J. E., *Heparin in the Treatment of Thrombosis,* Oxford University Press, London, New York, Toronto, 1946.

29. Landsteiner, K., *The Specificity of Serological Reactions,* Harvard University Press, Cambridge, Mass., 1947.

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Methylandrostenediol: A Non-Virilizing Derivative of Testosterone in Metastatic Cancer of the Breast.[*] (17840)

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Methylandrostenediol[‡] was chosen for clinical trial in patients with metastatic cancer of the breast because it was the first of a series of compounds tested which on bioassay showed renotropic effects on experimental hydronephrosis in female adult mice resembling those of testosterone propionate. The method and results of these bioassays will be published elsewhere(1). The marked degree of protection afforded the tubular epithelial cells by testosterone propionate and methylandrostenediol in experimental hydronephrosis is illustrated in Fig. 1. This renotropic property of methylandrostenediol is at present under investigation in human renal disorders. Testosterone, vinyl testosterone, cis testosterone, testosterone tocaptate and testosterone carbonate failed to give such renal protection.

Methylandrostenediol was given to 7 women with metastasizing cancer of the breast for periods of 1 to 7 months, and in doses varying from 25 mg in oil given intramuscularly to 100 mg per day intramuscularly in aqueous suspension. The latter dose was found to be clinically the most effective. Pellets of 60 mg implanted every 20 to 30 days were also used. The maximal total dose was 10 g given intramuscularly in aqueous suspension.[§]

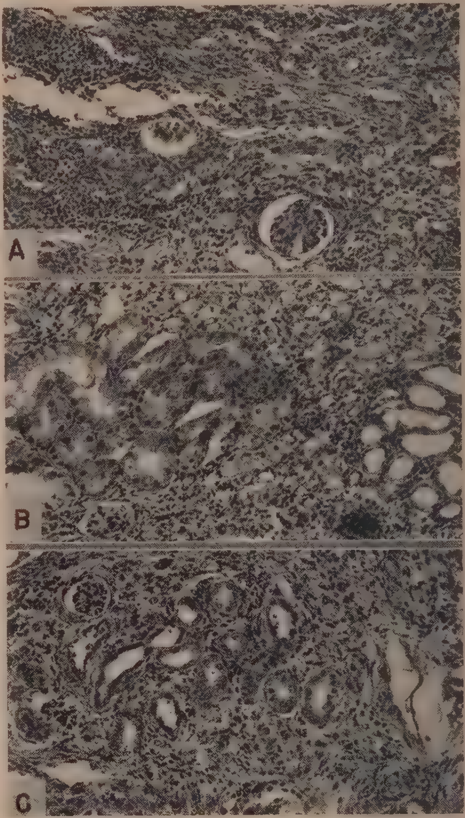

Fig. 1.

A. Fibrosis, round cell infiltration, and glomerular damage caused by unilateral ligation of the ureter lasting 30 days. This control animal received peanut oil. Magnification ×200.

B. Preservation and hypertrophy of tubular epithelium coexisting with destroyed tubules and other evidence of hydronephrosis in an animal treated like the control mouse (A) but receiving testosterone propionate. (Total dose 37.5 mg in 30 days). Magnification × 200.

C. Section from the hydronephrotic kidney of an animal receiving methylandrostenediol showing preservation of tubular epithelium. (Total dose 37.5 mg in 30 days). Magnification × 200.

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[†] The authors express their gratitude to the cooperating institutions: The Boston Dispensary Tumor Clinic, the Jewish Memorial Hospital, Roxbury, and the Holy Ghost Hospital, Cambridge.

[‡] Methylandrostenediol is a product of Organon Co., Inc., Nutley, N. J. Other steroids assayed in this work were obtained from the Ciba Co., Summit, N.J., the Merck Co., Rahway, N.J., the Schering Co., Bloomfield, N.J., and from Winthrop-Stearns, Inc., New York City.

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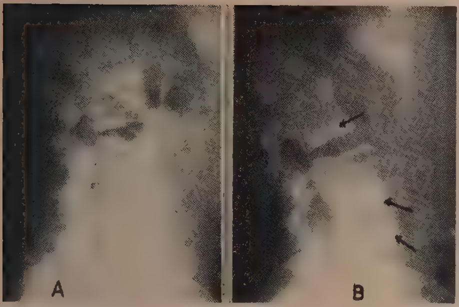

FIG. 2.
A. Patient G. M. X-ray of lumbar spine taken before methylandrostenediol therapy (12/8/48).
B. The same patient, after therapy (4/22/49), showing calcium deposition in lesions identified by arrows.

The patients were women ranging in age from 34 to 64 years, in whom cancer of the breast had been diagnosed as being of from 1 to 8 years duration prior to the initiation of methylandrostenediol therapy. Subjective improvement with varying degrees of euphoria and a sense of well-being occurred in all cases and lasted for from 1 to 7 months under therapy. Objective clear-cut calcification of bone lesions studied by X-ray was found in 2 patients and one is shown in Fig. 2 and 3. Soft tissue lesions regressed in 2 other instances under methylandrostenediol therapy. Studies of blood chemistry (phosphorus, calcium, alkaline phosphatase, NPN and total protein) showed trends comparable to those seen in the course of chemotherapy of breast cancer with testosterone and are illustrated in Fig. 4. There were instances of a drop in blood phosphorus, as well as fluctuations of blood calcium (without, so far, any cases of pathological hypercalcemia). Elevations of blood alkaline phosphatase were observed repeatedly following the administration of methylandrostenediol.

A metabolic study in one woman of 42 without cancer showed that methylandrostenediol has a protein anabolic effect of about 50% of that obtained with a similar dose (25 mg per day for 6 days) of testosterone propionate. These measurements were made by means of nitrogen, phosphorus, calcium and potassium balance studies on the metabolic ward of the Jewish Memorial Hospital. Six-day metabolic periods were alternated with control periods of 12 days. A constant diet was maintained throughout the experiment.

None of the women treated showed any virilizing or toxic signs. One patient who had grown a beard and acquired acne and a deep voice during testosterone therapy lost these symptoms under methylandrostenediol therapy, while she showed subjective as well as objective improvement of her breast cancer. (Fig. 2 and 3).

In summary, methylandrostenediol showed renotropic effects in bioassay resembling those of testosterone propionate. Our preliminary clinical observations seem to indicate that

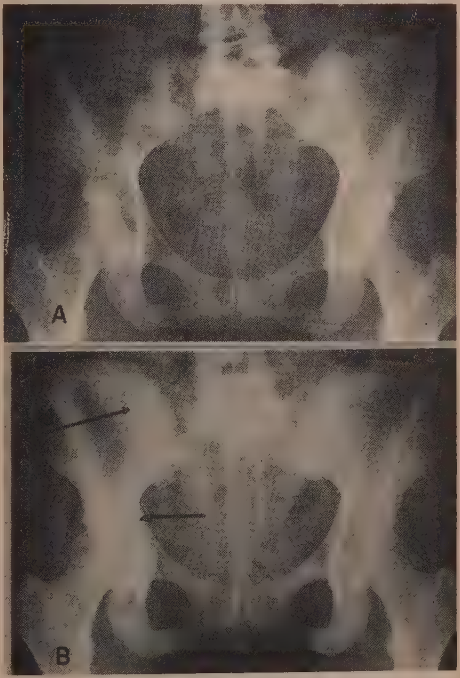

FIG. 3.
A. Patient G. M. X-ray of pelvis before methylandrostenediol therapy (12/8/48).
B. Same patient. X-ray of pelvis, showing calcification of lesions during methylandrostenediol therapy (4/22/49).

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§ At the time of correcting galley proofs (June 3, 1950) the maximal total dose given without undesirable effects was 28 g.

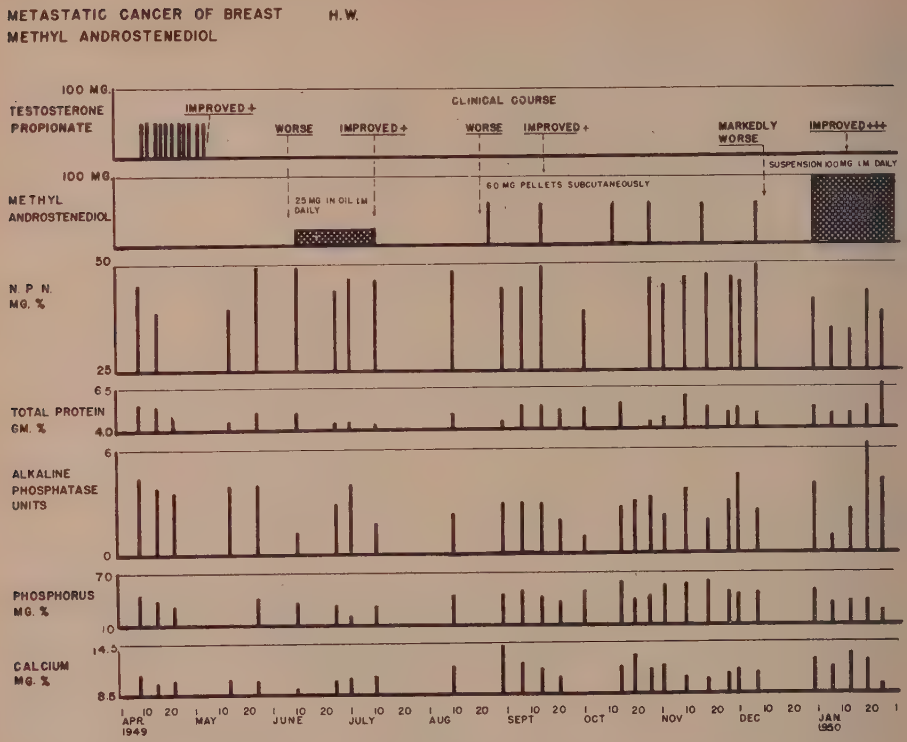

Fig. 4.

Chart of blood chemistry change as related to therapy and clinical changes. Patient H. W.

methylandrostenediol is a non-virilizing steroid hormone with moderate protein anabolic effect, and that it causes subjective well-being in patients with cancer of the breast. It did not bring about in our experience any of the undesirable biochemical phenomena such as water retention or hypercalcemia which often occur with testosterone. In at least 3 out of 7 cases of breast cancer objective regression of soft tissue and bone lesions was observed.

These brief clinical experiences do not justify conclusions as to the exact therapeutic value of this drug. However, they indicate the need for further critical evaluation on a larger scale.

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Alkaline Phosphatase Activity of Human Serum in Cancer.* (17841)

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From the Achelis Laboratory of the Lenox Hill Hospital, New York City.

Elevated serum alkaline phosphatase values are generally accepted as indicating either bone disease(1) or liver damage(2-4). The role alkaline phosphatases play in uncontrolled growth has been postulated by Potter (5,6). Potter and Liebel(7) have shown

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* Aided by a grant from the Frederick Kolb Fund.

TABLE I.
Statistical Analysis of Alkaline Phosphatase Activity of Human Serum. All values in mg %.

Substrate used	No. of patients	Range	Mean	Stand. Dev.
Adenosinetriphosphate				
(1) Normals	17	.5-3.1	2.2	.7
(2) Non-malignant	81	.1-6.4	2.2	1.2
(3) Cancer	51	1.2-21.0	6.2	5.7
(4) After removal of malignant tumor	28	.8-3.5	2.4	1.0
Yeast adenylic acid				
(1) Normals	52	.9-2.9	1.9	.5
(2) Non-malignant	161	.5-19.5	2.6	2.3
(3) Cancer	106	1.4-21.6	5.4	4.3
(4) After removal of malignant tumor	28	.9-3.5	2.4	.5
β-glycerolphosphate				
(1) Normals	24	1.4-3.7	2.3	.7
(2) Non-malignant	37	1.2-15.5	3.4	2.9
(3) Cancer	37	2.0-20.5	5.8	4.2
(4) After removal of malignant tumor	14	.7-3.4	2.5	.7

that in cancer tissue there exists, in high concentration, an enzyme capable of hydrolyzing ATP. Meister(8,9) reported that serum adenosinetriphosphatase hydrolyzes ATP with a maximum activity at pH 8.9. The enzyme is accelerated in liver disease and in patients with bony metastasis from prostatic carcinoma. We have found that similar dephosphorylation occurs with yeast adenylic acid and β-glycerolphosphate, and have studied the serum phosphatase activity using these substrates, in normal, in non-cancerous, and in individuals with neoplastic malignant disease.

Methods and materials. Substrates were adenosinetriphosphate, (*Tetra Sodium Salt* of ATP, Rohm and Haas, Phila., Pa.), yeast adenylic acid (Schwartz Laboratories, N. Y.), and β-glycerolphosphate (Eastman Kodak Company, Rochester, N. Y.), each in a concentration of 3.38 micromols per 2.5 ml of veronal-HCl buffer pH 8.9. This concentration gave optimum activity under our experimental conditions.

Procedure. 0.5 ml of non-hemolyzed serum is pipetted into each of 2 tubes. As a control one of these tubes is inactivated at 57°C for ½ hour. The control, the non-inactivated serum and the buffered substrate are brought to 37°C. 2.5 ml of substrate is added to each tube, the contents mixed, stoppered and incubated at 37°C for 2 hours. The inorganic phosphorus is determined by the method of Fiske and Subbarow(10). When ATP was used all determinations were made simultaneously to obviate errors due to spontaneous hydrolysis(11). Preliminary studies showed that dephosphorylation of the various substrates was proportional to the amount of serum used, and to the incubation period. The enzyme activity of the serum was not affected by refrigeration. Variations in the amount of phosphorus released in 2 hours were sufficiently great to establish a difference in enzyme activity between cancerous and non-cancerous individuals. The amount of phosphorus release selected as indicative of a significant increase in phosphatase activity for each of the substrates employed, (this value will be referred to as the S.I.)

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TABLE II.
Distribution of Patients with Malignant Tumors According to the Significant Increase.

Substrate used	Site of tumor	No. of cases	Significant above	Increase below
Adenosinetriphosphate	Liver and pancreas	6	6	0
	♂ G.U. System	6	6	0
	♀ ” ”	5	4	1
	Gastro-Intestinal Tract	17	8	9
	Breast	4	2	2
	Lung	5	2	3
	Misc.	8	5	3
Yeast Adenylic Acid	Liver and Pancreas	14	14	0
	♂ G.U. System	11	8	3
	♀ ” ”	8	6	2
	Gastro-Intestinal Tract	40	28	12
	Breast	11	9	2
	Lung	11	6	5
	Misc.	11	7	4
β-glycerolphosphate	Liver and Pancreas	4	4	0
	♂ G.U. System	5	3	2
	♀ ” ”	6	4	2
	Gastro-Intestinal Tract	11	8	3
	Breast	5	4	1
	Lung	1	1	0
	Misc.	5	5	0

was the mean plus twice the standard deviation of the normal group. The normals consisted of healthy laboratory workers, nurses and internes.

Results. In Table I, the results of our studies of phosphorus release in 2 hours using ATP, yeast adenylic acid and β-glycerolphosphate as substrates are recorded. With ATP the S.I. was found to be 3.6 mg%, 90 of 98 patients known to be free from neoplastic malignant disease had values below this figure. Of the 28 patients in whom the phosphatase was determined after removal of a malignant tumor only 2 showed a greater release than 3.6 mg%. Using yeast adenylic acid, the S.I. was found to be 2.9 mg%, and in 181 out of 213 non-cancerous subjects the value was below this figure. After removal of a malignant tumor, only 2 out of 28 patients showed a value greater than 2.9 mg%. Using β-glycerolphosphate substrate, the S.I. was 3.7 mg%. 54 of 61 of the non-cancerous subjects had a value below this figure. All of the 14 patients whose phosphatase was determined after removal of a malignant tumor showed a normal response. In individuals with some form of neoplastic malignant disease, the phosphatase was determined before surgical intervention or other treatment was instituted and the diagnosis was confirmed by biopsy or post mortem examination. These data are presented in Table II, and are classified according to the site of the tumor and whether the phosphatase activity is above or below the S.I.

Discussion. The sera of normal, the vast majority of non-cancerous individuals, and all cases of pregnancy showed a normal dephosphorylation. The main exceptions were those with diseases of the liver and pancreas, (the majority of whom were jaundiced) and those with endocrine disturbances, particularly thyroid disease. The S.I. of the cancer group was not influenced by the age or sex of the individual, nor by the site, type or degree of the malignancy. The greatest phosphorus release was found in those with tumors of the liver and pancreas, most of whom were jaundiced and those with malignancies of the genito-urinary system. In those with carcinomas of the gastro-intestinal tract showing normal phosphatase activity, the majority involved malignancy of the recto-sigmoid. In contrast over 92% of all the patients from whom a malignant tumor had been previously removed and were symptom free, the dephosphorylation of the various substrates was within normal limits. If all cases of known jaundice are excluded from our results, the same relationship between cancerous and non-cancerous individuals still holds true. The p value, as calculated by the statistical method of Fisher(12), of the normal, of the non-cancerous, and of patients after removal of a malignant tumor showed no difference, but was statistically significant when compared with that of the cancer group.

No specific reaction for adenosinetriphosphatase could be demonstrated. Yeast adenylic acid substrate gave the most consistent results, it is a stable compound, not subject to spontaneous hydrolysis in acid solutions and has a much lower S.I. than that of the other substrates. These factors might be of some value in the differential diagnosis of neoplastic malignant disease. Under our experimental conditions the dephosphorylation of the β-glycerolphosphate was lower than in the usual Bodansky procedure(13). No specificity or differentiation of serum alkaline phosphatase could be established, since the same serum gave similar dephosphorylation regardless of the substrate employed. The relationship of phosphorus release between cancerous and non-cancerous individuals was not influenced by substrate used.

Summary. The sera of normal and non-cancerous individuals have the same magnitude of dephosphorylation. In patients having a malignant tumor, phosphorus release of the serum was generally higher than the normal and non-cancerous group. The highest values were found in patients with tumors of the liver, pancreas and genito-urinary system. The sera of patients from whom a malignant tumor had been previously removed showed a phosphorus release which was identical with the normal and non-cancerous group.

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Histamine as Possible Chemical Mediator for Cutaneous Pain. Dual Pain Response to Histamine. (17842)

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This is the eighth in a series of studies that support the theory of histamine or a histamine-like substance as the chemical mediator for cutaneous pain, be it direct or referred(1-7). It has been demonstrated(7) that as little as 54 molecules of histamine (0.01 ml of 10^{-18} base) introduced into the superficial layers of the skin will cause painful responses, and that the intensity of the response is directly proportional to the concentration of histamine. The sensations considered previously were of the latent type only. In the present study both the immediate and latent responses to histamine in human subjects are considered.

Methods. Care of materiel. Scrupulous care was taken in the cleaning of the glassware, needles, etc. All glassware was cleaned in sulphuric acid potassium dichromate solution. The needles were placed in running water for 4 to 6 hours and then rinsed by forcing 10 cc of distilled water through them. After sterilization and before use, needles

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TABLE I.
Immediate Painful Response on Intradermal Injection of Histamine in Human Subjects.

			Pain on injection				
Conc.	No. subjects	No. samples	0	+	++	+++	% pos.
Saline	40	48	32	14	2	0	33
10^{-15}	26	37	6	25	6	0	84
10^{-7}	36	28	4	9	11	4	86
10^{-5}	33	30	0	6	9	15	100

TABLE II.
Delayed Painful Response to Intradermal Injection of Histamine in Human Subjects.

			Delayed pain response					
Conc.	No. subjects	No. samples	No. +	% +	Time onset, seconds*	Duration, min.†	No. with incr.	% with incr.
Saline	40	76	44	58	32	0.56	2	3
10^{-15}	26	61	52	85	33	1.6	8	13
10^{-7}	36	49	46	94	27	1.8	14	29
10^{-5}	33	44	42	95	16	3.5	34	79‡

* Considering only those in which there was a delayed response.
† Considering all samples.
‡ Considering 34 of 43 samples.

and syringes are rinsed in sterile saline 3 times. Histamine hydrochloride or phosphate as base was diluted in sterile saline made with chemically pure water.

Procedure for testing. The subjects were drawn mainly from the medical faculty of the University of Illinois and supplemented by second year medical students. The subjects were not told of the nature of the experiment other than that they would receive intradermal injections and that they were expected to describe any local effects sustained. The subjects were made comfortable in a reclining chair with foot stool. All external noises or interruptions were minimized. The subjects were told not to look at the injection site. Using a 27 gauge needle and a 2 cc syringe, the needle was gently inserted as superficially as possible, just burying the needle aperture. When it was ascertained that the pain from this insertion was over, about 0.01 ml was injected very slowly, producing a 2 to 3 mm wheal. No prompting by the operator was given with the following exception: in the low concentrations, many of the subjects would fail to voice the result during the time of injection. At this point the subject would be asked if he noted anything during injection. Comparisons with previous injections were also solicited. The results of each injection were timed by stop watch for a minimum of three minutes and recorded in the words of the subject.

Results. Forty subjects received a total of 230 injections. Both the immediate and latent sensations were studied.

Immediate response. In Table I the immediate responses to 143 injections are recorded. This number is less than the total number of injections because at the beginning of the study many individuals failed to voice the effect produced during the injection of the solutions. They stated that after the experiment that they expected some sort of sensation on injection, and therefore did not mention it. For this reason, the procedure of direct questioning of the subject immediately after injection was adopted. The intensity of the immediate painful response was judged by comparing the responses to various solutions injected in each individual subject. The grading was from 0 (no response) to 3+ (reserved for some exclamation of pain). The table thus represents the summation of individual experiments (0 to 3+) as well as a consideration of the responses as a whole (in percentages). From this table it is evident that the degree of response was directly related to the concentration of histamine. It is also noted that the highest number responding on injection (100%) was to the most concentrated solution of histamine (10^{-5}). The nature of the immediate response varied from a pricking, tingling sensation to acute pain, depending upon the concentration of histamine. Some described it as "being stuck with a needle" or "being burned" or "being stung by a bee or a nettle".

Latent response. The time of onset after injection, duration and the augmentation of the latent response is recorded in Table II. A little better than half (58%) gave a latent response following saline. When this occurred, it was relatively late after the injection (mean = 32 seconds) and was of very short duration (mean = 34 seconds). Only 2% or 3% showed an augmentation of this sensation. Following histamine injections practically all (94% and 95%) responded to the higher concentrations of histamine. What is equally significant is that the mean duration of this response was directly related to the concentration of histamine (1.8 minutes for 10^{-7} and 3.5 minutes for 10^{-5} dilution). Similarly, in the higher concentrations a greater percentage of subjects indicated an augmentation of the painful response. The time of onset of this latent response was shortened appreciably in the 10^{-5} dilution (mean = 16 seconds). In some instances the immediate and latent responses merged. The character of this sensation was described as pricking, stinging, tingling, burning, dull and sharp pain. Itching was noted with the higher concentrations(7). Sensations of cold, warmth or pressure were occasionally reported, but were not included in the tabulation of painful responses.

Considering the presence or absence of the latent painful sensation only, and combining results of this study with those of a previous one, 67 subjects are represented upon whom 408 injections were performed. These subjects represent both sexes, Negro, white and Asiatic races, and age ranges from 20 to 45 years (Table III). The percentage of subjects yielding a latent painful response is di-

TABLE III.
The Presence or Absence of the Delayed Painful Response in Human Subjects Following Histamine Injection.

			10^{-5}			10^{-6} to 10^{-8}			10^{-5} to 10^{-18}			Saline		
	No. subjects	No. samples	No. samples	No. +	% +	No. samples	No. +	% +	No. samples	No. +	% +	No. samples	No. +	% +
Exp. 1	27	178	10	10	100	64	58	91	44	31	70	60	21	35
,, 2	40	230	44	42	95	49	46	94	61	52	85	76	44	58
Total	67	408	54	52	96	113	104	92	105	83	79	136	65	48
Value of p				<.001			<.001		<.001					

rectly proportional to the concentration of histamine. Comparing the latent response of histamine to that of saline, the value of p in each concentration was less than .001, indicating its significance statistically.

Discussion. Under the conditions of this experiment, histamine injected into the superficial layers of the skin caused immediate painful sensations which usually disappeared promptly, but reoccurred after a lapse of time (latent or secondary responses). The intensity of the painful sensation and its duration was directly proportional to the concentration of histamine, while the time lapse between the primary and secondary response varied inversely with the concentration. Histamine when injected into the deeper layers of the skin fails to produce pain, but will cause a wheal and hyperemia. In two subjects having pronounced dermographia or skin allergies, the histamine pain response was diminished. The immediate response, especially in the lower concentrations, may not be elicited at a given point of inoculation, but may be noted at a site adjacent to it. This phenomenon is possibly related to the proximity of the injected histamine to the specialized pain receptor points. The latent response is most likely due to the summation of subthreshold impulses initiated by the diffusion of histamine. The relatively prolonged length of time for the appearance of this response excludes the time of conduction through nerves of varying diameter as a factor.

The high percentage of individuals reacting to saline injection, especially for the latent response, can in part be due to the fact that upon insertion of the needle, a certain amount of histamine is liberated. The fact that 58% of the subjects in this study as compared to 35% of the subjects in a previous study experienced delayed responses to saline may be explained by the fact that in this study the majority of the subjects were older and engaged in research and thus were more perceptive, whereas in the former study the majority were medical students. The failure of Lewis(8) and more recently Emmelin and Feldberg(9) to note pain after histamine injection is probably due to the method of pricking the histamine into the skin, and the concentration of histamine. By injecting histamine by means of needle and syringe, larger quantities are more easily delivered to the site of the specialized receptors of the skin and the fibers of the sensory nerves about them. The concentration of histamine in the skin is considered to be about 1:50,000(8). This is a sufficient concentration to cause all gradations of cutaneous pain. There is no histaminase in the skin(10).

Summary. 1. Histamine introduced into the superficial layers of the skin of human subjects causes an immediate as well as a latent painful sensation. 2. The intensity and the duration of the response are directly proportional to the concentration of histamine. 3. The lapse of time between the primary and the latent secondary response is inversely proportional to the concentration of histamine. 4. These findings give further support to the theory that histamine or a histamine-like substance may be the chemical mediator for cutaneous pain.

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Use of Ultraviolet Light and Fluorescent Dyes in Bacterial Counting.
(17843)

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It is the purpose of this preliminary paper to introduce a new, improved method of counting bacteria. The bacteria are stained with fluorescent dyes and counted in the Petroff-Hausser chamber and illuminated by a reflected ultraviolet light. Ultraviolet light and fluorescent dyes have been used previously in detecting tubercle bacilli(1,2) and protozoan parasites(3). Attention was focused upon the further use of ultraviolet light and fluorescent dyes in studying bacteria by observations noted in connection with the detection of cancer cells of vaginal smears as performed by Friedman(4) in this institution. The clarity of the mixed bacterial flora suggested the applicability of this technique to the bacterial counting chamber.

Apparatus. The General Electric AH4 mercury vapor lamp was mounted in a lamphousing. This lamp was operated from a special transformer. A UG4 Schott filter (Fish-Schurman Corporation, New York City) 6 m in thickness was placed in front of the lamphousing lens system. The smooth surface of the substage glass microscope mirror should be replaced by an aluminum faced mirror as the latter has a higher reflectivity for ultraviolet light. A protective Eastman Kodak Wratten 2A filter is inserted into a standard monocular microscope. The Wratten filter is used for the purpose of filtering out any stray ultraviolet rays while simultaneously allowing the visible fluorescent light to pass freely through the eyepiece. (Fig. 1) A Petroff-Hausser counting chamber with Neubauer ruling No. 4101-A was used in this work. It was difficult to pick up the Neubauer rulings on the counting chamber from this source of light. Therefore, a special Petroff-Hausser counting chamber was obtained with the Neubauer rulings etched with deeper lines.

Method.[*] A bacterial suspension to be counted is drawn up into the Thoma dilution pipette for red blood cells to the 0.5 mark. This is diluted to the 101 mark above the bulb with a 1/1000 dilution of the desired fluorescent dye, thus making a 1:200 dilution in the bulb itself. Should the growth of organisms be scant, a dilution of 1:20 is made by means of the No. 3392 Thoma White Cell pipette. The pipette is shaken in the usual manner to break up clumps and to make an even distribution of the bacteria throughout the suspension. The suspension is left for 5 minutes to allow the bacteria to take up the stain. Four to 6 drops of the suspension are allowed to escape through the

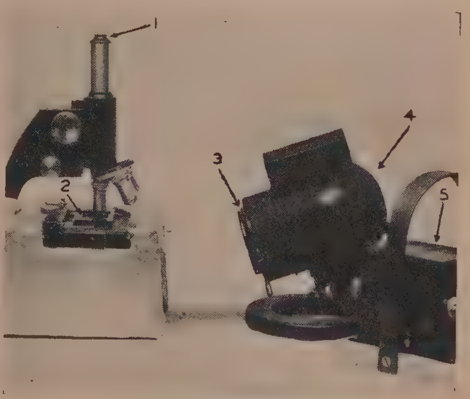

Fig. 1.

1. Microscope ocular with Wratten 2A filter inserted.
2. Bacterial counter.
3. UG4 Schott filter.
4. Lamphousing with General Electric AH4 mercury vapor lamp.
5. Transformer.

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TABLE I
Response of Selected Organisms to Fluorescent Dyes when Exposed to Ultra-Violet Light

	Sarcina lutea	Strep. viridence	Strep. pyogenes	Staph albus	Staph. aureus	Proteus X-19	C. diptheriae mitis	E. coli	A. aerogenes	Shigella shiga	Sal. paratyphi B	Sal. paratyphi A
Acid Fuchsin												
Acridine Orange	3*		3	2	1	2	2	1	1			2
Acridine Yellow	1	3	2		3	2	3	3	3	1	1	3
Acriflavin	2	2	2		3			3	3	2	2	2
Alizarin Red	1	1	1							1	1	
Auramine O	1	1	1	1	1	1	1	1	1	1	1	1
Berberine Sulfate	1	1	1	1	1	1	1	1	1	1	1	1
Congo Red												
Eosin Y				3	3		2	3	3			3
Fluorescein												
Methylene Blue												
Neutral Red												
Phosphine 3R	1	1	1	3	3	3	3	3	3	1	1	2
Primulin Yellow	1	1	1	2	2	1	1	1	1	1	1	2
Proflavin	3	3	3	3	3	3	3	3	3	3	3	3
Rhodamine B	1	1	1	1	1	1	1	1	1	1	1	1
Thioflavin S	1	1	1	3	3	3	3	3	3	1	1	3

*1. Excellent 2. Good 3. Fair Blank–Unsatisfactory

tip of the pipette and discarded after which the counting chamber is loaded in the routine manner so that the ruled area is covered without overflow into the moat. The room should be moderately darkened for best results. Four to 6 minutes must be allowed for the mercury lamp unit to reach a uniform ultraviolet light production. The count is then made. When viewed through the ocular of the microscope, the bacteria are seen to fluoresce brightly and distinctly against a black background.

Results. 17 different fluorescent dyes were investigated in varying pH of 5.0 through 7.0. (Table I). Fluorescent dyes are photosensitive and should be protected from visible light when not in use. The following microscopic organisms (The first 12 organisms were obtained through the courtesy of the Department of Bacteriology, University of Chicago) have been used and have been found to be very satisfactorily counted by this technic:

1. *Salmonella paratyphi* A; 2. *Salmonella paratyphi* B; 3. *Shigella shiga;* 4. *Escherichia coli;* 5. *Aerobacter aerogenes;* 6. *Staphylococcus aureus;* 7. *Staphylococcus albus;* 8. *Sarcina lutea;* 9. Proteus X-19; 10. *Corynebactor diphtheria* (mitis); 11. *Streptococcus pyrogenes;* 12. *Streptococcus viridans*; 13. *Trichomonas vaginalis;* 14. *Candida albicans.*

There were 3 dyes in this series which were superior with the organisms studied; namely: Auramine-O, Rhodamine-B, and Berberine Sulfate. A fourth dye, Primulin Yellow was found to vary from good to excellent depending upon the bacterial strain. (Table I). Berberine sulfate was preferred in this study because of the relationship of color and background contrast. Numerous bacterial counts were made using both visible and fluorescent light sources on identical cultures. The comparative value is very simply illustrated by the following example. Four samples from a thoroughly suspended culture of *E. coli* were taken in immediate succession. The fluorescent dye was used as the diluent. The counts were made first with visible light and then the identical squares were counted with the ultraviolet light. The average count using visible light was 192,400,000 bacteria per milliliter. The average count under ultraviolet light was 307,000,000 bacteria per milliliter. The single counts made under visible light ranged from 160,000,000 bacteria per milliliter to 240,000,000 bacteria per milliliter, whereas the same fields counted making use of the ultraviolet light ran from 300,000,000 to 320,000,000 bacteria per milliliter. Thus more bacteria could be counted in equal dilutions with the ultraviolet light and fluorescent dyes than with ordinary light. The counts made using this new technique averaged less than one minute for actual counting time as compared with several minutes when the standard visible light was employed.

Summary. 1. The use of ultraviolet light and fluorescent dyes as an adjunct to the Petroff-Hausser bacterial counting chamber technique is reported.

2. This method has made bacterial counts more accurate because of clarity, ease, and rapidity.

3. Berberine Sulfate was the dye of preference although Auramine-O and Rhodamine-B were gauged equally as good.

4. Twelve strains of bacteria, one protozoa, and one fungi were used in testing this procedure.

5. All 14 organisms gave excellent fluorescent reaction to 3 dyes and 11 strains gave excellent results to a fourth dye.

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Response of the Pig to APF, B_{12} and B_{12b}. (17844)

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Using a corn-peanut meal ration Cunha *et al.*(1) showed that an animal protein factor supplement (Lederle) and crude concentrates (charcoal and fuller's earth) of B_{12} differed in their effect on the pig. The objective of this experiment was to determine what the effect of a relatively pure concentrate of B_{12} and B_{12b} is when given orally or when injected and to determine whether or not the effect is different from that of APF.

Materials and methods. The control ration used consisted of corn, peanut meal, minerals, and the known vitamins which the pig has been shown to need as previously described by Cunha *et al.*(1) Four 8-week-old pigs, equalized with respect to sex, condition, weight, thriftiness and previous dietary history were allotted to each lot. The feeding period was for 40 days. The pigs were housed and fed on concrete floors which were washed once daily.

Results and comments. The data obtained in this trial (Table I) show that vitamin B_{12} (a mixture of B_{12} and B_{12b}), fed either orally or injected did not significantly affect the rate of growth when added to the corn-peanut meal basal ration for a feeding period of 40 days. This confirms earlier data by Cunha *et al.*(1) when concentrates (charcoal and fuller's earth) of B_{12} were fed orally. The pigs injected with B_{12} in lot 2 did not gain as well as the pigs on the basal ration. The pigs in lot 3, fed APF and injected with B_{12}, did not gain as rapidly as the pigs fed APF only in lot 5. Thus, in the 2 lots of pigs, where B_{12} was injected, it seemed to decrease the rate of gain about 0.1 lb. daily.

A diarrhea was observed during the first 2 weeks of the trial in the pigs on the basal ration and periodically throughout the trial in the pigs in lots 2 and 4 where vitamin B_{12} was used. No diarrhea was observed in lots 3 and 5 where APF was added to the ration. Thus, these and other observations obtained with this basal ration at this Station, show that APF contains some factor or factors which prevents a diarrhea (semi-liquid yellowish feces) in pigs. The APF supplement (lot No. 8108-109 prepared by drying the mycelium from deep aerobic cultures of *Streptomyces aureofaciens* together with some filter aid) was found to contain 3 mg of aureomycin per gram by microbiological assay at Lederle Laboratories. Jukes *et al.*(2) and Cunha *et al.*(3) showed that either aureomycin or APF prevented diarrhea with

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TABLE I.
Effect of APF, B_{12}, and B_{12b} on the Pig.

Lot No.	Ration fed	Avg starting wt, lb	Avg final wt, lb	Avg daily gain, lb
1	Basal	19.1	49.1	0.75
2	” + B_{12} injected*	19.4	45.8	0.66
3	” + ” ” +APF†	20.0	66.0	1.15
4	” + ” orally‡	20.1	49.3	0.73
5	” + APF†	18.9	68.9	1.25

* B_{12} conc. which contained approximately equal parts of a relatively pure mixture of vit. B_{12} and B_{12b} (had at least 10 mg of B_{12} activity per gram of solids) obtained from Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N.Y. B_{12} mixture was inj. peritoneally at the rate of 5 γ per kilo body wt once weekly.

† Lederle APF supplement (lot No. 8108-109) fed at a level of 1000 g per 100 lb of feed.

‡ The B_{12} and B_{12b} mixture was added to the feed at a level of 20 γ per lb of feed.

pigs fed a ration similar to that used in lots 2 and 4. Since APF contains aureomycin, and the aureomycin alone prevents the diarrhea, it is probable that aureomycin itself is responsible for preventing the diarrhea obtained with the pigs in this trial. The pigs fed APF had much smoother hair coats and showed more bloom than the pigs in the other lots where APF was not included.

The pigs fed APF (lots 3 and 5) gained at a considerably faster rate than the pigs fed the basal ration with or without B_{12} and B_{12b}. These data show that APF contains a factor(s) other than B_{12} or B_{12b} which is of importance for the pig. Jukes *et al.*(2) showed that aureomycin was of considerable benefit in increasing the rate of gain of pigs when added to a corn-peanut meal ration similar to that used in this trial. Therefore, aureomycin accounted for at least a part of the growth promoting activity of the APF used in this trial since it contained aureomycin.

Summary. A mixture of B_{12} and B_{12b} was of no benefit for growth when administered by injection or orally to the pig during a 40-day period under the conditions of this experiment. APF prevented a diarrhea which occurred periodically with the other pigs on experiment. APF fed pigs exhibited a smoother hair coat and more bloom. Further evidence was obtained to show that APF contains a factor(s) other than B_{12} or B_{12b}.

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Agglutination of Mammalian Erythrocytes by Newcastle Disease Virus.*

(17845)

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The ability of Newcastle disease virus (NDV) to cause the agglutination of mammalian erythrocytes was studied as part of the problem of characterizing strains of the virus. Burnet(1), Clark and Nagler(2), Brandly *et al.*(3), and Chu(4) had reported agglutination of certain mammalian cells by NDV. Different strains of the virus were used by each of these investigators and since some of their findings were in disagreement, the possibility of the strain being the source of such differences was suggested.

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1. Burnet, F. M., *Aust. J. Exp. Biol. and Med. Sci.*, 1942, v20, 81.

2. Clark, Ellen and Nagler, F. P. O., *Aust. J. Exp. Biol. and Med. Sci.*, 1943, v21, 103.

In our studies, 31 strains of NDV isolated from natural outbreaks over a period of 15 years in this country, Canada, England and Italy were examined. Erythrocytes of 11 mammalian species were tested including those of man, guinea pig, mouse, fox, dog, horse, bison, cow, goat, sheep and elk.

Materials. Virus strains were selected from among those received by the NDV repository at the University of Wisconsin. Most of the strains had undergone from 2 to 8 egg passages and 6 strains were available after various egg passages ranging up to 60 passages. Individual histories and certain characters of these strains, *i.e.,* chicken pathogenicity and heat tolerance, were described by Hanson(5). For this paper the tri-symbol designation of each strain, *i.e.,* KD-NJ-1945 indicating its code, place and time of origin will serve to identify the strain and its history. A prefix (T) or (H) signifies that the strain was obtained from a turkey or human source, and if not so specified the strain was derived from a chicken. Mammalian red blood cells were collected in 4.0% sodium citrate, centrifuged and washed 3 times in physiological saline. In all but one case cells were used within 3 to 5 days after drawing. A 1% suspension of cells in saline was used for the hemagglutination test. In most instances the agglutinability of blood cell samples from 5 or more individuals was determined for each species of animal. When possible the individuals were chosen to include divergent types of a species. Human blood samples included O, A and B blood types. Cattle blood was obtained from the Holstein, Guernsey, Jersey and Brown Swiss breeds. Some of the tests were made with cattle blood of known cell antigen types, supplied by Dr. Clyde Stormont, Department of Genetics, University of Wisconsin and some with cells obtained at various times during the estrous cycle. Cells of Toggenberg and Saanen breeds of goats and Suffolk sheep were used. Guinea pig blood was drawn from commercial stock pigs; mouse blood from strain H white mice originally from Rockefeller Institute stock and from the inbred mouse strain Z obtained from Iowa State College, Ames, Iowa. Silver foxes, mongrel dogs and Belgian horses supplied the blood from those species. Blood of bison and elk *Cervus canadensis nelsoni* came from the Wichita Mountains Wildlife Refuge, Cache, Okla., and was furnished by Dr. M. R. Irwin, Department of Genetics, University of Wisconsin. The bison blood was in two instances several weeks old at the time of testing.

Methods. The procedure of testing for hemagglutination activity (HA) was simple. The virus was used in the form of clear allanto-amnionic fluid pools from 4 to 5 13-day-old infected embryonating eggs. A simplification of the Salk hemagglutination technique was followed using only 2 dilutions of the virus, 1:10 and 1:20. To a volume of 0.5 ml of the diluted virus was added 0.25 m of the desired red blood cell suspension, and the test incubated at room temperature, about 25°C, for 30 to 45 minutes. Agglutination was determined by the pattern of sedimentation on the bottom of the tube. Suitable controls were set up with each test, *i.e.,* red blood cell, normal allantoic and normal amnionic fluids.

Results. Red blood cells from all tested individuals of 5 mammalian species, man, guinea pig, mouse, dog and bison, were agglutinated by all strains of the virus. On the other hand all samples of fox erythrocytes failed to be agglutinated by any strain of virus. The results of variably susceptible species are presented in Table I. Five strains of virus, KD-NJ-1944, 46967-Mich-1946, Lan-Wis-1947, 11914-Calif-1944, Herts-Eng-1933, did not agglutinate any of these cells. Cells from the 5 mammalian species were agglutinated by some strains of virus, not

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3. Brandly, C. A., Moses, H. E., Jungherr, E. L. and Jones, E. Elizabeth, *Am. J. Vet. Res.,* 1946, v7, 289.

4. Chu, C. M., *J. Hyg.,* 1948, v46, 239.

5. Hanson, R. P., Thesis submitted for Ph. D. degree, University of Wisconsin Library, 1949.

TABLE I.
Hemagglutinating Activity of 21 Strains of Newcastle Disease Virus for 5 Species of Mammals.

	Mammalian erythrocytes*				
	Horse	Cow	Goat	Sheep	Elk
4F-Mass-1945	—	(—)	(—)	—	—
Levine-NY-1945	(+)	—	++	—	+
(T)Mayer-NY-1945	(—)	—	—	—	++
Roakin-NJ-1946	—	++	++	+	—
23-Iowa-1946	—	++	++	++	+
(T)547-Minn-1947	—	—	(—)	—	—
31747-Mo-1947	—	—	(+)	—	—
L-Kans-1948	(++)	—	(+)	—	++
M-Kans-1948	++	—	+	—	++
(T)Henry-Wis-1948	—	—	++	—	+
CG179-Calif-1944	—	—	—	—	++
670-Utah-1948	—	—	+		
Wat-Ont-1948	++	++	++	++	+
Ber-Ont-1948	++	—	++	++	++
(T)Peer-Sask-1948	(++)	++	++	+	+
All-Que-1948	(—)	—	(+)	—	++
Cle-Ont-1948	++	++	++	++	++
Lab-Ont-1948	—	(++)	++	++	++
Herr-Ont-1948	++	(++)	++	++	++
McVey-Ont-1948	++	(++)	++	++	++
M-Italy-1945	(++)	(++)	++	++	

* At least 5 individuals of each species.
+, ++, Degree of agglutination.
—, Lack of agglutination.
( ), Variation in agglutination, majority of individuals giving reaction noted.

TABLE II.
Agglutination by NDV of the Erythrocytes of 8 Species of Mammals.

	Investigator				
Mammal	Burnet*	Clark*	Brandly*	Chu*	Wisconsin†
Man	+	+	+	+	100
Guinea pig	+	+	+	+	100
Mouse	+			+	100
Dog			+	+	100
Horse	0	0	0	0§	54‡
Cow		+	+‡	0§	32‡
Goat	+	+		0§	50‡
Sheep		+	0	0§	40

* Expressed as agglutinated, +, or not agglutinated, 0.
† Expressed as % of virus strains demonstrating agglutination of the erythrocytes.
‡ Differences observed between samples of red blood cells from different donors.
§ Agglutinated only when purified virus was used.

agglutinated by others and inconsistently agglutinated by a few.

Comparative tests of 25 strains of the virus with horse, cow, goat and sheep cells showed that 6 strains agglutinated none of the 4 species, 13 agglutinated 1 to 3, and 6 agglutinated all species. Of the 25 strains of virus 19 agglutinated goat cells, 13 agglutinated horse cells, 10 agglutinated cow cells and 10 agglutinated sheep cells.

Table II summarizes selected data from the work of previous investigators and compares it with our results The ability of NDV to agglutinate certain mammalian red blood cells appears to be a distinct strain character

In addition to the strain of virus other factors influence the interaction of virus and red blood cells and must be considered in the evaluation of the effect of strains. Observations were made of the effect of variability of red blood cells among a given species, the effect of the pH of the reactants, and the effect of egg passage upon the strain individuality.

Brandly(3) reported that the cells of some cows were agglutinated while those of others gave negative results. Consequently all our tests were set up to give a limited survey of cell susceptibility among individuals by testing 5 or more individuals of each species. Only 3 species of mammals, horse, cow and goat, yielded blood cells which differed in their agglutinability when tested by a single strain of the virus. Of 17 strains agglutinating horse erythrocytes 9 of these strains agglutinated only those of certain individuals. Four of 10 strains which agglutinated cow red blood cells agglutinated only certain samples and of 22 strains which agglutinated goat red blood cells 6 failed to clump the cells of all individuals.

The differential susceptibility of blood cells of individual cows was examined in some detail. It appeared to be unrelated to certain cell antigens, to the stage of estrus during which it was drawn, to lactation, or to breed. Samples of red blood cells obtained at diverse intervals from an individual cow yielded essentially the same agglutination spectrum.

The effect of pH upon the agglutination of red blood cells was investigated. In studying influenza virus Magill(6) had observed this to be a factor. The pH of the standard reactants in the HA test as determined with a Coleman potentiometer was 7.8. Preparations of Sorensen's phosphate buffered saline of pH values ranging from 5.6 to 8.2 were used as diluent for the test. The pH of the medium employed influenced the susceptibility of the erythrocytes to agglutination by NDV. The number of strains agglutinating cow erythrocytes increased from 35 to 82% when the pH was reduced from 7.8 to 6. While certain qualitative differences in agglutination were observed when the pH of goat erythrocyte suspensions was altered, the number of agglutinating strains remained about 70%. Sheep red blood cells, like those of the cow, were much more susceptible to agglutination at pH 6, 66% of the strains agglutinating at that pH as compared to 28% at pH 7.8. The effect of temperatures above or below approximately 25°C on hemagglutination was not ascertained.

Most of the strains were tested after only 3 to 6 egg passages. Two strains, Hertfordshire-Eng-1933 and KD-NJ-1945, available at both the 5th and 55th passage and at 5th and the 60th passage levels, respectively, showed no significant change in agglutinability occurring as a consequence of egg passage. After 6 passages strains 23-Iowa-1945, Milano-Italy-1945, and Leavenworth-Kans-1948 also showed no differences in their agglutinative activity.

Summary and conclusions. The ability of NDV to induce agglutination of mammalian erythrocytes of certain species is a character possessed by some but not all strains of the virus. The erythrocytes of certain individuals of such species as the cow, the horse, and the sheep are more susceptible than those of others to agglutination by NDV. Agglutination of mammalian red blood cells, as a rule, may be recorded accurately as the per cent of individuals whose cells are susceptible to the action of a given strain of NDV and not as a uniformly negative or positive result. This percentage of erythrocyte susceptible individuals may range from zero in some species to 100% in others but in most species lies between these extremes so that its consideration is warranted in every instance. It was demonstrated that the pH may affect the agglutination of mammalian red blood cells and, consequently, alter the interpretation of the test. If suitable controls of the variables of the test are included and the source of red blood cells is properly selected many strains of NDV may be differentiated by their mammalian agglutination range or spectrum.

The mechanism underlying these observations has not been investigated. The most obvious explanation predicates differences in the agglutinative activity of the virus strains, possibly associated with variable levels of enzyme production or activity. Chu would hold accountable differences in the levels of one or more inhibitors of hemagglutination. In either case, the activity or lack of activity

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6. Magill, T. P. and Sugg, J. V., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 89.

seems to be associated with lines or strains of virus. The differing hemagglutinative susceptibility of individual donors suggests that the mechanisms involved are rather complex.

The phenomenon of agglutination of mammalian erythrocytes has practical application in maintaining the identity of stock strains of virus and possibly also in determining probable intermingling of the viruses of live vaccines and wild strains in field outbreaks of Newcastle disease.

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Effect of Mercurials on Kidney Adenosine Triphosphatase Activity.*

(17846)

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Mercurial diuretics are capable of producing renal tubular damage. Preliminary experiments(1) have shown that kidney adenosine triphosphatase (ATP-ase) activity is inhibited by these compounds. Since very little is known about the nature of nephrotoxic action of drugs, it seemed of interest to study the possible relationship between the inhibitory effect of mercurials on kidney ATP-ase activity and the renal tubular injury produced by these drugs.

Material and methods. White rats of both sexes weighing approximately 200 g were used in this study. They were sacrificed by a blow on the head. The kidneys were homogenized at ice-water temperature in an all glass homogenizer. ATP-ase activity was determined by the method of DuBois and Potter(2). All enzyme determinations were done in quadruplicate. Inorganic phosphate was determined by the method of Fiske and Subbarow(3) using a Coleman spectrophotometer. ATP was obtained as the barium salt from the Nutritional Biochemicals Corporation, Cleveland, Ohio. The solution of the sodium salt was prepared just prior to the performance of the experiment. The mersalyl used was Salyrgan-Theophylline-Winthrop. The amount of theophylline in this preparation was found not to affect ATP-ase activity in the concentrations employed. In the *in vivo* experiments, one kidney of each rat was homogenized; the other was fixed in 10% formalin for microscopic studies. The ATP-ase activity of normal rat kidneys was determined simultaneously with the determinations done on kidneys of injected animals.

Results. Table I shows the effect of the addition of mersalyl and mercuric chloride on the ATP-ase activity of homogenized rat kidneys. Inhibition was observed consistently following the addition of these compounds in concentrations as low as 0.0001 M. The inorganic mercurial produced a greater inhibition at any given concentration than the organic compound.

TABLE I.
Effect of Mercurials on ATP-ase Activity of Homogenized Rat Kidneys.

	ATP-ase activity* per mg of kidney Final concentration of added drug		
Drug	0	0.0001M	0.00001M
Mersalyl	57	51	57
	58	50	55
Mercuric Chloride	57	35	54
	58	38	52

* μg of phosphorus liberated per hr.

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* We are grateful to Dr. E. E. Muirhead for carrying out the microscopic studies

1. Goth, A., Holman, J., and O'Dell, V., *Fed. Proc.*, 1950, in press.

2. DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, 1943, v150, 185.

3. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, v66, 375.

TABLE II.
ATP-ase Activity of Rat Kidneys Following Intramuscular Injection of Mersalyl.

Exp. No.	Dose, mg/kg	Time after inj., hr	ATP-ase activity*		% difference from mean of controls†		Microscopic tubular damage‡
			per mg of kidney	per total kidney, $\times 10^{-3}$	per mg of kidney	per total kidney	
1.	5	4	60	44	0	—19	0
			61	49	+ 2	—10	0
		24	57	43	— 5	—21	0
			56	43	— 7	—21	0
2.	10	4	54	59	—10	+ 8	0
			54	56	—10	+ 3	0
		24	62	57	+ 3	+ 5	+
			60	45	0	—17	+
		48	53	40	—12	—26	+
			54	43	—10	—21	+
		72	62	63	+ 3	+16	+
			55	57	— 8	+ 5	+
3.	50	4	62	50	+ 3	— 8	++++
			61	59	+ 2	+ 8	++++
		24	44	33	—27	—39	++++
			44	49	—27	—10	++++
		48	44	39	—27	—28	++++
			41	41	—32	—25	++++
		72	24	34	—60	—38	++++

* μg of phosphorus liberated per hour. ATP-ase activity per mg of kidney multiplied by the weight of the kidney in g gives total activity per kidney.

† The mean ATP-ase activity of 19 normal rat kidneys was 60 per mg of kidney and 54 per total kidney with the standard deviations of 8 and 10% respectively.

‡ Microscopic tubular damage: 0 = none; + = occasional tubules necrotic; ++++ = majority of tubules necrotic.

Table II shows the effect of the injection of varying doses of mersalyl on the kidney ATP-ase activity of rats sacrificed at varying intervals following the injection. Regardless of the dose injected, there was no significant change in ATP-ase activity as early as 4 hours following the injection of the mercurial. Inhibition was evident in 24 hours and subsequently, especially in the group which received 50 mg per kg of mersalyl. In this group, marked renal tubular damage was present 4 hours following the injection. At that stage no significant changes in ATP-ase activity could be demonstrated.

The decreases in ATP-ase activity could not be due to dilution caused by the swelling of the kidneys, since the total ATP-ase activity of the kidney was decreased also. Furthermore, a number of kidney ATP-ase determinations in which enzyme activity was calculated on the basis of dry weight indicated also that there was an actual depletion of ATP-splitting enzyme.

Discussion. The results of this study show that mersalyl and mercuric chloride inhibit kidney ATP-ase activity *in vitro*. It is also evident from this study that the injection of mersalyl into rats leads to a decrease in kidney ATP-ase activity. Since microscopic studies demonstrated a marked renal tubular damage which preceded the enzymatic changes, it appears that the decrease in ATP-ase activity is a consequence and not a cause of tubular damage. It is possible that the inhibition of other enzymes may precede the development of kidney damage, but it is also possible that the "disorganization of the protoplasm which may or may not precede the inactivation of numerous enzymes" as suggested by Bernheim(4) represents the cause of renal injury in acute mercury poisoning.

Conclusions. 1. Mersalyl and mercuric chloride were found to inhibit ATP-ase activity of homogenized rat kidneys. 2. Following the intramuscular injection of various

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4. Bernheim, F., The Interaction of Drugs and Cell Catalysts, Burgess Publishing Co., Minneapolis, 1948, p43.

doses of mersalyl into rats, renal ATP-ase activity was unchanged in 4 hours; it was decreased after 24 hours. 3. Simultaneous microscopic and enzymatic studies suggest that the decrease in ATP-ase activity is a result and not a cause of renal injury produced by mersalyl.

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Acetylcholine Content of Tyrode Solution Perfused Through Muscles as Affected by Calcium and Procaine Hydrochloride.* (17847)

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There appear to be many similarities between muscle fibrillation caused by denervation and fibrillation produced by calcium lack(1,2,3,4,5). These experiments were performed in order to examine the possible rôle of acetylcholine in fibrillating dog muscle caused by these two conditions.

Method. A week to 10 days before an experiment a dog's left sciatic nerve was severed aseptically. On the morning of the experiment the animal was lightly anesthetized with pentothal sodium and prepared for cannulation of the right iliac artery and vein. The following solutions were in readiness: mammalian Tyrode solution containing sufficient heparin to provide the animal with a total of 10 mg/kg; Tyrode solution containing eserine in a concentration of 1:100,000. Two Ca^{++} free Tyrode solutions were also prepared and to one of them was added 1 millimol of procaine hydrochloride per liter. Provision was made for warming these solutions to body temperature by passage through a glass coil immersed in a tank maintained at about 40°C. Electromyograms were obtained from wire recordings made continuously throughout the experiment. We have recently described this technic and full details are readily available in another journal(6). The method is recommended as the wire recorder permits reproduction of the electrical activity of the muscle for photography at a later time when the experimenter is not preoccupied with operative and other technics. The standard procedure was as follows: the right sciatic nerve was cut, a concentric needle was inserted into the right gastrocnemius, and with the amplifier, wire recorder and loud speaker turned on, the right iliac artery was perfused with Tyrode solution containing heparin. Usually no electrical activity from the muscle was heard on the speaker. The iliac vein was then cannulated and the sanguinous perfusate collected. The muscle was then perfused with Tyrode solution containing eserine 1:100,000, calcium free Tyrode solution and lastly, calcium free Tyrode solution containing procaine hydrochloride 1 millimol per liter. Samples of perfusate were collected in separate flasks, additional eserine added, and the perfusates stored in the ice box for further use. A complete recording of the electrical activity of the perfused muscles was obtained. The whole procedure was then repeated on the denervated side and again separate eserinized samples of perfusate were

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6. Dunn, A. L., Bennett, A. L., and McIntyre, A. R., *J. Applied Physiol.*, 1948, v1, 402.

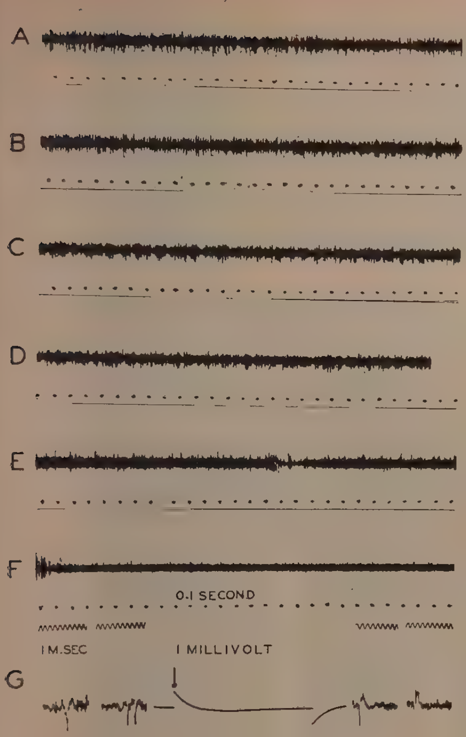

Fig. 1.

collected and stored.

Experimental results. The wire recording of muscle activity was fed into a suitable amplifier connected to a cathode ray oscilloscope and photographed on moving bromide paper. Fig. 1 contains representative samples of the recordings obtained from one of the 4 satisfactory experiments completed without complications. It will be seen from examination of the upper 4 records (A,B,C, D) that the electrical activity of the denervated muscle remained virtually constant throughout the experiment. However, long continued perfusion of a denervated muscle with Ca^{++} free Tyrode solution containing procaine hydrochloride usually caused a gradual decrease in electrical activity (higher concentrations of procaine hydrochloride, 10 millimolar, may interrupt the fibrillation relatively quickly). In contrast it was found that the electrical activity of the innervated muscle aroused by perfusion with Ca^{++} free Tyrode solution (record E) promptly ceased when 1 millimolar procaine hydrochloride was added to the perfusing fluid (record F). The last record (G) shows single spikes from denervated muscle (left) and single spikes from innervated muscle while being perfused with Ca^{++} free Tyrode solution (right).

The acetylcholine content of the perfusates. The perfusates were rendered protein free by treatment with trichloroacetic acid; after filtration they were neutralized with $NaHCO_3$. The protein free filtrates were divided into two portions. One of each pair was rendered alkaline (pH 8-8.5), briefly heated, cooled and neutralized with CO_2. Finally all filtrates including those hydrolyzed by the above procedure were tested for ACh using the inhibition of the heart of *Venus mercenaria* as an indicator. Records from 4 clam hearts are presented in Fig. 2. The upper row consists of cardiograms obtained from a series of controls in which Tyrode solution, Tyrode solution treated with trichloroacetic acid and then neutralized, Ca^{++} free Tyrode solution and Ca^{++} free Tyrode solution containing 3 millimols of procaine hydrochloride (3 times the concentration used for muscle perfusion) were added successively in quantities of one milliliter to the sea water at A,B,C, and D respectively. The heart was not washed between these 4 tests. The initial bath volume was 35 cc. At E, 1 cc of sea water containing 0.1 γ of ACh bromide was added. Between F and G 0.1 γ of ACh in 1 cc of sea water containing 3 millimols of procaine hydrochloride was gradually added and after washing with sea water, 0.1 γ of ACh in 1 cc of sea water was added at H. It will be seen that although the amplitude of the contractions gradually declines, the effect of a final concentration of 0.0025 γ of ACh per cc of sea water was plainly visible. The first record in the second row was obtained from another heart in which 1 cc of protein free Tyrode solution, obtained after perfusion through an intact muscle *at rest* was added to the bath at I. Note the increase in amplitude of the heart contraction (see discussion below). The record at J was obtained after the addition to

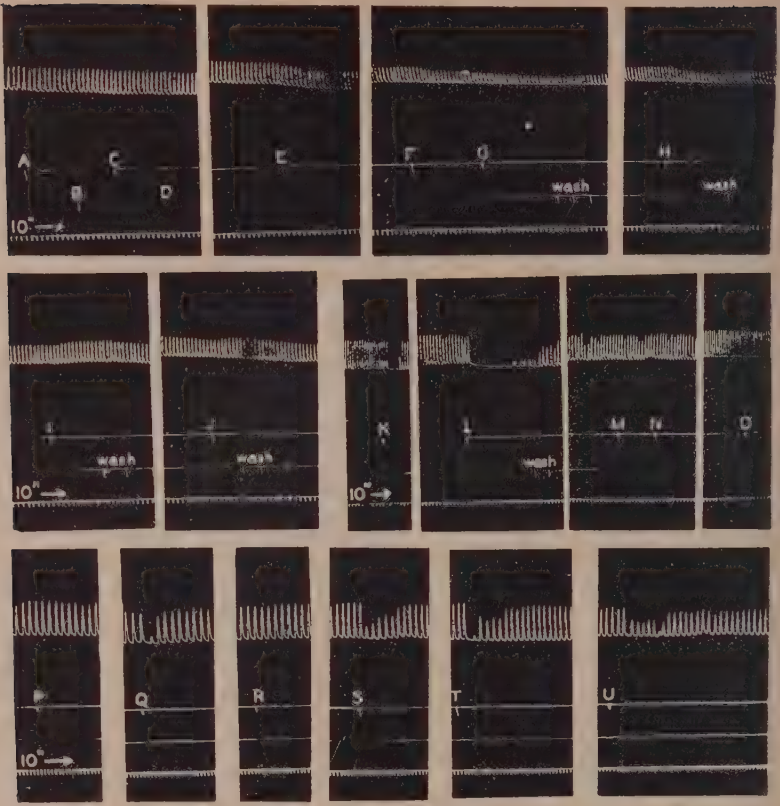

FIG. 2.

the water bath of 1 cc of *hydrolyzed* Ca^{++} free filtrate, collected during intense muscle-activity. (It will be noted that again the amplitude of contraction was slightly augmented, see discussion.) The remaining records on the second row were obtained from a third clam heart and show (K) the inhibition following the addition of 1 cc of Ca^{++} free Tyrode solution containing 1 γ of ACh. At L is shown the effect of 1 cc of Ca^{++} free Tyrode solution after perfusion through an active innervated muscle. The next record shows the effect obtained when filtrate prepared from Ca^{++} free perfusate containing procaine hydrochloride, and obtained during the cessation of muscle activity, was gradually added to the bath between M and N. In the next record, at O, a similar filtrate but obtained after muscle activity had ceased was added to the bath. Note the absence of cardiac inhibition. The remaining records (bottom row) were obtained respectively from: the addition of 1 cc of Tyrode solution containing a large excess of K^+, (3 times the normal content) to the bath (P); the addition of 1 cc of Tyrode solution containing a microgram of ACh (Q). R shows the absence of inhibition obtained from a *hydrolyzed* protein free perfusate obtained during active fibrillation of a denervated muscle; (compare this record with J). The remaining 3 records were obtained from solutions perfused through the same denervated fibrillating muscle. S, shows the presence of a cardio-inhibitor in Tyrode solution, T, the continued presence of this substance in a Ca^{++} free Tyrode perfusate and U, the continued presence of this cardio-inhibitor in a Tyrode solution containing 1 millimol of procaine hydrochloride.

Discussion. The high degree of specificity of *Venus mercenaria* hearts for ACh has been established by many(7,8,9,10,11,12,13). The nature of the substance frequently seen in hydrolyzed perfusates from muscles, which causes the augmentation of cardiac amplitude, seen in Fig. 2 at I,J and to a less extent at R, has not been determined. It occurs in protein free filtrates prepared by trichloroacetic acid preparation and survives heating at pH 8. The possibility of the presence of ATP in the perfusates, so prepared, cannot be excluded. Our experiments reveal that ACh appears in the eserinized perfusates from muscles undergoing spontaneous fibrillation induced by a deficiency of calcium or as a consequence of denervation. They also show that procaine arrests spontaneous muscle activity due to calcium lack in the innervated preparation, but has no immediate effect upon the spontaneous activity of denervated muscle.

It might be thought that procaine arrests spontaneous activity caused by calcium lack by simply blocking ACh access to muscle effectors. This conclusion is, however, erroneous because the cessation of spontaneous activity occurs within a few seconds and at that time the muscle will respond to indirect excitation, and the failure of procaine to arrest this spontaneous fibrillation of the denervated muscle appears to preclude the possibility of block. Furthermore the work of Jaco and Wood(14) clearly demonstrated that the intra-arterial injection of procaine (in comparable concentrations to those used by us) did not interrupt indirect excitation. These workers found that when a muscle was indirectly stimulated by maximal spike shocks applied to the nerve the intra-arterial administration of prostigmine greatly increased the amplitude of the muscle contraction; however, when procaine was then injected intra-arterially the normal muscle response to indirect stimulation promptly returned and continued without further decrement. They also found block of indirect excitability of the muscle induced by the intra-arterial injection of 25 γ of ACh was prevented by the intra-arterial injection of cocaine. They point out that curarine, unlike procaine, fails to antagonize prostigmine. Hence the actions of procaine and curarine at the neuro-muscular junction differ: procaine depresses the production of acetylcholine at the neuromuscular junction; curarine, on the other hand, blocks acetylcholine. Consequently the failure of indirect excitation caused by procaine reported by Harvey(15) is brought about by separate mechanisms. It is believed that the observations reported here and those of others all are capable of explanation by the following hypothesis. Under normal conditions acetylcholine production at the neuromuscular junction results as a consequence of depolarization of the membrane at the termination of the motor axon and the destruction of acetylcholine is associated with the repolarization of the same structure. Any acetylcholine that "escapes" from the immediate vicinity of the motor-nerve endings is destroyed by the cholinesterase associated with the "palisade region" described by Couteaux(16) and possibly by the cholinesterase of the R.B. cells(17). Under these conditions repetitive responses of muscle to a single nerve stimulus are impossible, neither can spontaneous

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muscle activity occur. In the presence of eserine or prostigmine any ACh that escapes destruction as a consequence of repolarization of the axon membrane accumulates and results in: (A) a larger muscle response to single indirect stimuli as described by Jaco and Wood (see above), (B) repetitive muscle responses as found by Eccles, Katz and Kuffler(18) and (C) neuromuscular block as described by Briscoe(19). These three phenomena can be duplicated by the intra-arterial injection of increasing concentrations of ACh. Jaco and Wood (see above) showed that ACh block was inhibited by cocaine and this effect is readily understandable when it is remembered that cocaine inhibits the depolarization of nerve(20) and hence not only diminishes the production but also augments the rate of destruction of ACh. The cause of spontaneous muscle activity in normal preparations exposed to calcium free solutions is, according to this hypothesis, the result of the accumulation of ACh as a consequence of partial depolarization of the motor axon terminal membrane resulting from the withdrawal of calcium ions. Direct measurements of the resting end-plate potential are difficult; a decrease in resting potential of nerve upon exposure to calcium free solutions has been observed by us. Kuffler (l.c.) also has reported negative resting potentials developing in muscles immersed in calcium free solutions and augmentation of muscle responses to single indirect stimuli, with the appearance of repetitive muscle responses and finally block. This is the same sequence of events which occurs when increasing concentrations of ACh are applied to the neuromuscular junction(21). The cholinesterase content of denervated muscle is not markedly lower than that of normal muscle(22) hence the increase in sensitivity of denervated muscle to ACh is not explainable on the basis of a lack of this enzyme, and some other explanation is required. It is believed that the hypothesis presented fills this need. In conclusion it should be pointed out, that with larger concentrations, or more prolonged exposure to 1 millimolar procaine, there may be direct effects upon muscles; and prolonged exposure to low calcium concentrations may also affect the muscles; under these abnormal conditions the hypothesis would not apply.

Summary. 1. Dogs' denervated fibrillating muscles when perfused with Tyrode solution, Ca^{++} free Tyrode solution, or Ca^{++} free Tyrode solution containing 1 millimol of procaine hydrochloride per liter, continue to fibrillate and the perfusate from such muscles contains a heat labile material capable of inhibiting the heart of *Venus mercenaria in vitro.*

2. Intact muscle perfused with Tyrode solution does not usually exhibit electrical activity and the perfusate from such inactive muscles does not inhibit the heart of *Venus mercenaria.*

3. Innervated muscle perfused with eserinized Ca^{++} free Tyrode solution displays electrical activity similar to the fibrillation of denervation and the perfusate from such muscles contains a heat labile material capable of inhibiting the heart of *Venus mercenaria in vitro.*

4. Perfusates subjected to alkaline hydrolysis obtained from denervated fibrillating muscle, or from innervated muscle rendered active by perfusion with Ca^{++} free solutions, do not contain a cardio-inhibitor substance when tested for such an inhibitor on the heart of *Venus mercenaria in vitro;* these solutions often augment the contractions of the isolated clam heart.

5. Electrical activity of innervated muscle perfused with Ca^{++} free Tyrode solution ceases when procaine hydrochloride is added to the perfusing fluid and the perfusate from the muscle then contains no heat labile cardio-inhibiting substance.

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6. A new hypothesis is discussed in which it is postulated that motor nerve endings at rest are in part responsible for the removal of acetylcholine at the neuromuscular junction.

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"Spontaneous" Ovulation in the Rabbit Following Combined Estrogen-Progesterone Treatment. (17848)

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Unlike the human and a large majority of other mammals, the rabbit seldom, if ever, ovulates spontaneously; normally ovulation occurs only after neurogenic stimulation of the hypophysis attendant on coitus. Estrogen has been reported to facilitate the release of ovulating hormone from the rabbit hypophysis in response to copper salts(1), to coitus during pseudopregnancy(2), and to mechanical stimulation of the vagina(3), but progesterone has always been considered inhibitory to ovulation in this species(4,5). In such widely unrelated spontaneously-ovulating forms as the rat(6,7,8) the hen(9) and probably the monkey(10), however, ovulation has been stimulated by the appropriately timed injection of progesterone. Everett(11) has suggested that once the critical factor of timing is understood, progesterone facilitation of LH release may be disclosed in other species. We have recently found that progesterone administration, independently of extrinsic estrogen, conditions the estrous rabbit in such a way that mechanical stimulation of the vagina induces ovulation(12). The present work reveals that, in a significant number of rabbits, combined treatment with estrogen and progesterone dispenses with the requirement of a coital stimulus and allows ovulation to occur "spontaneously."

This preliminary study employed 30 mature female rabbits, all apparently in estrus as judged by hyperemia of the vaginal orifice. To avoid the possibility of mechanical stimulation the vagina was not examined after the first hormone injection. The estrogen used was α-estradiol benzoate.* The rabbits were injected subcutaneously on 2 consecutive mornings with doses of 85 μg estrogen in 0.1 ml peanut oil and/or 2 mg progesterone in 0.2 ml peanut oil. In the animals receiving both hormones the progesterone was administered within 4 hours after the second estrogen injection. The ovaries were examined for ruptured follicles at laparotomy about 48 hours after the last injection.

The results are summarized in Table I. It is apparent that whereas neither estrogen nor progesterone alone was followed by ovulation, 4 out of 10 rabbits ovulated after the combined treatment.

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* We are grateful to the Schering Corporation, Bloomfield, N.J., for generously supplying the estradiol benzoate and progesterone.

TABLE I.
Summary of Experimental Results.

		Treatment		Results		
No. of rabbits	Avg wt, kg	Estradiol benzoate μg	Progesterone mg	Ovulated	Failed to ovulate	% ovulated
10	3.5	2 × 85	—	0	10	0
10	3.4	—	2	0	10	0
10	3.2	2 × 85	2	4	6	40

been effective in facilitating ovulation have all been spontaneous ovulators, though at least in the rat a neurogenic timing factor is involved in the LH-release mechanism (13,14,8). The rabbit is the first form in which, to our knowledge, a species normally requiring a coital stimulus has been converted to a "spontaneously ovulating" type by hormone administration. In this respect it resembles the persistent-estrous rat, in which spontaneous ovulation can be restored by progesterone treatment(6). To what extent in the rabbit progesterone operates through nervous mechanisms requires further study. Addition of this animal to the growing list of species in which progesterone stimulates ovulation, increases the likelihood that progesterone facilitation may be a fairly general phenomenon.

Summary. Following treatment with estrogen and progesterone 4/10 rabbits ovulated spontaneously, *i.e.,* without the coital stimulus normally required to induce LH release from the hypophysis in this species.

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Effect of Cortisone on Some Reactions of Hypersensitivity in Laboratory Animals.* (17849)

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The effects of ACTH and cortisone which have been reported in the case of a number of different disease states known or thought to involve hypersensitivity of tissues(1-3) have suggested that these agents may influence the manifestations of antigen-antibody reactions *in vivo* or of bacterial hypersensitivity. Accordingly, a study was instituted of the effect of cortisone on a number of these reactions of immunity and hypersensitivity in experimental animals.

Procedure. 1. *Effect of cortisone treatment on the tuberculin skin reaction in sensitized rabbits and guinea pigs.* a. A total of 18 rabbits of approximately 2.2 kg were infected intradermally with 0.15 mg of tubercle bacilli (BCG). After 3 weeks it was established that cutaneous sensitivity to tuberculin (OT) and to cytoplasmic particles (CP)

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TABLE I.
Tuberculin Test in Rabbits Sensitized with BCG.

			Skin test with OT Volume in mm^3—48 hr				Skin test with CP Volume in mm^3—48 hr			
Test period	Group	No.	Neg.	to 200	201-400	>400	Neg.	to 200	201-400	>400
Preliminary	Control	10	1	4	2	3	1	4	4	1
	Cort.	8	0	4	2	2	0	5	1	2
Test	Control	10	0	7	3		0	4	4	2
	Cort.	8	8	0	0		8	0	0	0

prepared from BCG organisms by high speed centrifugation(4) was present in all of the rabbits. Cortisone was injected intramuscularly into 8 rabbits twice daily at a total daily dose of 34 mg per rabbit. A fall in the total number of circulating lymphocytes was noted in all 8 rabbits within 1 day after beginning the injections. After 3½ days of cortisone treatment all the rabbits were tested by intradermal injections of 0.1 cc of OT 1:10 and CP (1 mg/cc). Readings of the volume of the intradermal inflammation (5) were made after 48 hours, after which the cortisone was discontinued. It was found (Table I) that the rabbits receiving cortisone did not exhibit the cutaneous evidence of sensitivity to proteins of the tubercle bacillus which they had shown in the preliminary tests.

b. Guinea pigs of approximately 360 g were infected intradermally with 0.15 mg of tubercle bacilli (BCG). After 3 weeks skin sensitivity to OT and CP was established. Twenty of these animals were given cortisone totalling 11 mg per day, injected intramuscularly twice daily for 6 days. Blood eosinophile counts made before and during administration of cortisone showed a sharp fall in the total number of these cells. On the third day of cortisone treatment all the guinea pigs were retested. Since the guinea pigs were multicolored the area of the dermal reaction could not be read with satisfactory accuracy. The intensity of the inflammatory response is therefore presented in terms of mm of thickening of the skin at the site of injection. These results (Table II) showed that of 20 guinea pigs under the influence of cortisone 17 and 18 failed to show positive reactions to OT and CP respectively, and 3 and 2 showed slight reactions. The entire group was again tested with the same dilutions of OT and CP 3 to 6 days after the last injection of cortisone, and dermal reactions now appeared again at the sites of the new tests. Some days after the cessation of cortisone therapy ulcers appeared in a number of the cortisone-treated guinea pigs at the sites of skin tests made during the course of cortisone treatment, and which had been negative at that time.

c. Seventeen guinea pigs sensitized with tubercle bacilli, 8 on their fourth day of treatment with cortisone and 9 controls, were given intraperitoneal injections of 0.6 cc of a preparation of OT which had been dialyzed, lyophilized and reconstituted to 5 times its original concentration. Within a few hours of this injection all the guinea pigs which had not received cortisone appeared to be ill, showing diarrhea, listlessness, lowering of body temperature and drooping of the eyelids so that the palpebral fissure was only 3 or 4 mm. The guinea pigs receiving cortisone remained well and active. Within 48 hours 7 of the 9 guinea pigs of the control group were dead, whereas the 8 cortisone-treated animals were alive and well. The adrenal glands of the control animals were removed at autopsy and were found grossly to be hemorrhagic in varying degrees, from a few small, distinct hemorrhages to a confluent hemorrhagic mass. The 2 remaining control animals were sacrificed and their adrenal glands also showed gross hemorrhages. For histologic study 2 guinea pigs of the cortisone group were sacrificed after 48 hours and their adrenal glands found

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grossly to be small and free of hemorrhages. The remaining animals of the cortisone group were sacrificed after 4 more days of apparent good health. Their adrenals were found to be normal on gross examination.

II. *Arthus reaction in rabbits.* Normal horse serum (NHS 1.0 cc) was injected subcutaneously into 19 rabbits of approximately 2.2 kg at weekly intervals for 4 weeks. After the fifth injection of NHS a local lesion appeared in most of the animals at the site of the injection, and an inflammatory reaction could be elicited after the injection of 0.1 cc of NHS intradermally. This latter reaction usually reached its height in 24 hours. Part of the group of rabbits was then given 34 mg of cortisone daily in 2 injections for a total of 5 days. On the third day after cortisone treatment was begun, another subcutaneous injection of NHS was given to all the rabbits and the thickness of the local lesion which appeared subsequently at the site of injection was measured, as well as the reaction to a skin test of 0.1 cc of NHS placed on the opposite side of the animal. Neither the extent of the local Arthus reaction nor the presence or intensity of the skin test reaction appeared to be affected by the administration of cortisone (Table III).

III. *Antibody production.* All of the rabbits involved in the tuberculin sensitivity experiment were injected subcutaneously with 2 cc of a mixture of sheep erythrocytes (50% suspension) and *Shigella paradysenteriae* (10 mg) one day after the injection of cortisone had begun. Blood was drawn from these animals after 3, 4, 6 and 8 days, and the serum tested for sheep red cell hemolysins and dysentery agglutinins. The rising curves of serum antibody titers against both antigens did not show significant differences between the group of cortisone-treated and control animals, although there was a tendency for higher titers to appear in the sera of the control group.

IV. *Anaphylaxis.* In one experiment guinea pigs received semi-weekly subcutaneous injections of 0.5 cc NHS for 4 weeks. Of the 14 animals 9 were then given varying doses of cortisone twice daily for 2½ days. Four hours after the last injection of cortisone all

TABLE II.

Tuberculin Test in Guinea Pigs Sensitized with BCG.

			Intensity of reaction—thickening of skin in mm											
			OT (1:20)				CP (2 mg/cc)				OT (1:10)			
Test period	Group	No.	Neg.	to 1	1-2	>2	Neg.	to 1	1-2	>2	Neg.	to 1	1-2	>2
Preliminary	Control	20	4	8	7	1	0	5	14	1				
	Cort.	21	5	8	7	1	1	8	12					
Test	Control	19	1	6	7	5	0	7	9	3	0	5	6	8
	Cort.	20	17	2	1		18	2			18	1	1	

TABLE III.
Arthus Reaction in Cortisone-Treated Guinea Pigs and Controls.

			Skin test readings Volume in mm^3—24 hr				Arthus reaction in mm —24 hr			
Test period	Group	No.	Neg.	1-200	201-400	>400	Trace	1-4	5-8	>8
Preliminary	Control	9	0	6	2	1		1	5	3
	Cort.	9	0	2	3	4	1	1	4	3
Test	Control	9	0	6	3			1	3	5
	Cort.	9	1	3	4	1		6	3	

the animals were given 1.5 to 2.0 cc of NHS by intracardiac injection. Shock occurred in all of the animals, and the number of deaths in the respective groups was not significantly different. In this experiment, which was the first one of this study, the range of dose of cortisone was considerably lower than that adopted later.

Discussion. The data presented here indicate that cortisone can suppress the manifestations of some of the reactions of hypersensitivity in experimental animals. Under the conditions of these experiments, in the case of rabbits and guinea pigs sensitized to tubercle bacilli, the cutaneous sensitivity of both species to OT, and the systemic shock of guinea pigs, were suppressed while the animals were under treatment with cortisone. This effect was not demonstrated in the case of anaphylaxis in the guinea pig nor of the local Arthus reaction in the rabbit. The latter findings are consistent with observations made by Leger(6) in anaphylaxis and Fischel(7) in the Arthus reaction in animals treated with ACTH. These contrasting results again point to some difference in the mechanism of the antigen-antibody reactions *in vivo* such as the Arthus phenomenon and anaphylaxis on the one hand, and the bacterial type of hypersensitivity such as the tuberculin reaction on the other hand. The mechanism of the action of cortisone in suppressing these reactions is not understood. In the cortisone-treated animals no reaction had been observed 24 or 48 hours after the intradermal injection of skin test doses; however, at some of these sites of injection ulcers were noted some days after the cessation of cortisone therapy. There would seem to be two alternative hypotheses for this sequence of events; first, that the cortisone does not actually prevent injury of the hypersensitive cell, but merely delays the objective manifestation of that injury and, second, that residual antigen remaining at the site 72 hours after the injection (a day after the last injection of cortisone) could then proceed to act as in the control animals.

Summary. In rabbits and guinea pigs sensitized with tubercle bacilli (BCG) dermal reactivity to Old Tuberculin and to cytoplasmic particles from tubercle bacilli and systemic shock produced by intraperitoneal injection of OT could be suppressed by treatment with cortisone. Four days after cessation of treatment the skin test again became positive.

Cortisone did not prevent anaphylactic shock in guinea pigs sensitized with normal horse serum nor did it exert any influence on either the appearance of the Arthus reaction in rabbits injected with normal horse serum or on the skin test reaction to an intradermal injection of normal horse serum in these rabbits.

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Daily Administration of Massive Oral Doses of Thonzylamine Hydrochloride. (17850)

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Now that certain of the newer antihistamine drugs are being sold directly to the public, the question has been raised in some circles whether they are safe for mass consumption. In order to supply additional information regarding the safety of these drugs, we report in the following paragraphs on the daily administration of massive oral doses of one of the antihistamine drugs, Thonzylamine hydrochloride.[†]

Experimental and results. Six young male dogs, inoculated against distemper and maintained on a stock diet, were observed for one month and then placed on test. Two dogs were maintained as untreated controls; 2 were given 20 mg and 2 were given 40 mg of Thonzylamine Hydrochloride per kg, orally each day. The animals were kept on test over a period of 26 to 52 weeks. Complete blood counts and urinalyses for reducing substances and albumin were performed monthly while weights were recorded weekly. During the test period all animals were in apparent good health and displayed none of the signs of spasticity or CNS excitation observed with other antihistamines at equal or lower dose levels(1,2). The findings (Table I) disclose no significant difference (Analysis of Variance and "t" test) between treated and control animals. Growth rates were not suppressed. The hematologic findings remained within the normal range specified by Downey (3) and urinalyses were consistently negative in all animals. At the conclusion of the study gross and microscopic examination of tissues disclosed no organ pathology.[‡]

Discussion. It has been reported that doses as high as 200 mg/kg of Thonzylamine

TABLE I.
Findings in Dogs Fed Massive Doses of Thonzylamine Hydrochloride.

	Untreated		Fed Thonzylamine				Normal data (Downey)
			20 mg/kg		40 mg/kg		
	Control No. 1	Control No. 2	No. 1	No. 2	No. 1	No. 2	Means (Limits)
Erythrocytes ($\times 10^6$)*	7.37	6.99	6.63	7.40	6.94	7.22	6.0 (5.6-7.6)
Leukocytes ($\times 10^3$)	8.36	10.51	9.42	7.78	9.57	9.33	11.17 (5.65-19.2)
Lymphocytes (%)	38	37	36	37	38	36	20 (9.34)
Large mononuclear cells (%)	1	2	1	2	2	2	4 (1-10)
Polymorphonuclear cells (%)	58	59	60	61	57	60	74 (56-87)
Eosinophiles (%)	2	2	2	2	3	2	2 (0-7)
Basophiles (%)	0	0	0	0	0	0	0 (Rare)
Initial wt, kg	12.4	15.2	15.2	12.7	6.4	7.3	
Final wt, kg	16.7	21.7	21.7	19.9	10.0	10.9	
Gain in wt, %	34.7	42.8	42.8	56.7	56.3	49.3	

* Mean values given for hematological data.

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[*] We are indebted to Joseph Mirsky for invaluable assistance.

[†] Originally reported under the name Neohetramine and currently sold also under the trade name "Anahist", Thonzylamine is 2-(N-dimethylaminoethyl-N-p-methoxybenzyl)-aminopyrimidine monohydrochloride.

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[‡] We are indebted to Dr. William Antopol of the New York Beth Israel Hospital for the preparation and examination of tissue sections employed in this study.

hydrochloride could be fed to weanling rats over protracted periods of time with no evidence of toxicity(4,5). Our present findings extend the earlier work to include a larger animal species and are of special significance because of the large dose administered daily for a period as long as one year. In view of the lack of toxicity in experimental animals it is not surprising to find that the drug is well tolerated in man(6-10).

Summary. Thonzylamine hydrochloride was administered to dogs at doses of 20 and 40 mg/kg, p. o., daily for a period of 6 months to one year. The animals grew at a normal rate and showed no evidence of blood dyscrasia throughout the experimental period. At necropsy there was no sign of gross or microscopic organ pathology.

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Assay of Methylcholanthrene-Induced Mammary Tumors of Mice for the Mammary Tumor Milk Agent.* (17851)

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It has been demonstrated that carcinogenic hydrocarbons induce mammary tumors in certain inbred strains of mice(1-4). Not only were these cancers induced in stocks in which the incidence of spontaneous mammary tumors is high, but also in strains with a low incidence which do not possess the mammary tumor milk agent. While the development of spontaneous mammary cancer in mice is usually dependent upon the combined influence of the milk agent and hormonal factors in animals of appropriate genetic susceptibility(5), mammary tumors were induced by the action of chemical carcinogens in mice of susceptible strains from which the milk agent had been excluded by foster nursing(6). However, tumors have appeared earlier and in higher frequency in mice of identical genetic constitution when the milk agent was present(6) and breeding resulted in a more precocious development of induced tumors(7,3). Dmochowski and Orr (8) recently reported that the milk agent could not be demonstrated in extracts of

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* This investigation has been aided by grants from the American Cancer Society, Inc., upon recommendation of the Committee on Growth of the National Research Council, the National Cancer Institute, U. S. Public Health Service, and the Cancer Research Fund of the Graduate School of the University of Minnesota.

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TABLE I.
Results on the Assay of 4 Tumors, Induced in Mice Without the Milk Agent, for the Presence of the Agent in Tumor Extracts. The incidence in the control test animals was 1.4%.

Tumor tested, No.	Dose, g equiv.	No. injected	% Ca.	Avg age in days	
				Cancer	Non-ca.
176	2×10^{-2}	28	3.6	223	563
176	10^{-3}	35	0		493
185	10^{-2}	18	0		573
185	10^{-3}	25	0		391
174	10^{-2}	41	2.4	268	484
174	10^{-4}	26	3.8	528	510
161	10^{-2}	21	0		416
161	2×10^{-3}	40	0		490
161	10^{-3}	18	0		447
Total		252	1.2		

tumors induced with methylcholanthrene in females of the IF and C57 black strains, nor in extracts of tumors induced in males by the combined action of methylcholanthrene and estrone. Both strains are relatively non-susceptible to spontaneous mammary tumors and do not carry the milk agent. When mammary tumors were induced in males of the high mammary cancer C3H stock by the hydrocarbon and estrone, extracts of these tumors were found to contain the milk agent. The authors concluded that the carcinogen did not cause the agent to appear in mice which did not possess it before treatment.

For the present study mammary tumors were induced by methylcholanthrene in breeding females of subline 212 of the dilute brown stock which did not have the mammary tumor milk agent (line D_2b—agent excluded by foster nursing). Animals were skin painted 3 times a week for 6 weeks with a 0.25% solution of methylcholanthrene in benzene, as described previously(3). Mice of subline 212 are susceptible to the development of either spontaneous or induced mammary tumors(7,9), whereas mice of the fostered line (D_2b) have given a low spontaneous incidence (unpublished data).

Four induced mammary tumors were tested for the presence of the milk agent. The preparation of the extracts for injection was similar for each tumor. Tissue was ground with sand in a mortar and extracted with distilled water to give a 10% suspension. Extracts were centrifuged for 10 minutes at approximately 2500 r.p.m., following which supernatants were removed and used at the concentrations given in Table I. Fractions were injected intraperitoneally and doses are expressed as the amount derived from the original tissue on a weight basis. Thus, a dose of 10^{-2} g equivalents represents the amount from 10^{-2} g of wet tissue in one cc. This method of biological assay has proved very satisfactory for the detection of the milk agent when present in very low concentrations in extracts of tissues(10,9).

Test animals were ZBC females, 21-26 days of age at the time of injection, which lack the milk agent and usually develop mammary tumors only when it is administered. These mice are back-cross hybrids produced by reciprocal matings between animals of the fostered A and Z (C3H) stocks. The incidence of mammary tumors in breeding ZBC females and their mothers, numbering over 2,000, has been 1.4%.

Three mammary tumors (1.2%) developed in 252 mice which received extracts of the 4 induced tumors (Table I). Sixty-two other animals were injected but are not included in the tabulations since they failed to survive for 223 days, at which time the first tumor was noticed. Thus, the mammary tumor milk agent could not be demonstrated in the extracts of 4 carcinogen-induced mammary tumors from mice of the dilute brown stock

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from which the milk agent had been eliminated by foster-nursing.

Few mammary tumors develop in mice of strains susceptible to spontaneous tumors if they do not have the mammary tumor milk agent, and the incidence cannot be significantly increased by the injection of estrogenic hormones(11,12). As opposed to these findings, mice of strains both susceptible and relatively resistant to the development of spontaneous mammary cancer have developed tumors following treatment with methylcholanthrene. These observations indicate that there is no positive correlation between the inherited susceptibility to spontaneous and to carcinogen-induced tumors(3,4). In fact, mice of different sublines of the same inbred stock may not show the same response. Breeding females of one subline of the C57 black stock revealed no induced mammary tumors(3) although when animals of the same line obtained the milk agent by nursing the incidence of spontaneous mammary tumors was at least 40% (unpublished data). Mice of another subline of the same strain, with a lower incidence of spontaneous tumors when they possess the milk agent, developed a few carcinogen-induced mammary tumors when females without the agent were maintained as either virgins or breeders and tumors appeared in the males when they were treated with both estrone and methylcholanthrene(4).

In the present study the extracts of 4 mammary tumors induced in the absence of the milk agent in mice susceptible (demonstrable when the agent is present) to the development of spontaneous mammary tumors were found to be free of the agent. Thus, the action of the carcinogen was not dependent upon the presence of a milk agent nor did it induce the genesis of an agent. Squamous metaplasia is characteristic of carcinogen-induced mammary adenocarcinomas and the tumors tested in this study had areas of keratinized stratified squamous epithelium. Dmochowski and Orr(4,8) observed that the degree of squamous metaplasia was greater in induced tumors developing in the low susceptible C57 black stock than in males of the high cancer C3H strain. The microscopic structure of mammary tumors that developed in mice free of the milk agent was similar to that of the carcinogen-induced tumors(13).

Summary. The mammary tumor milk agent was not demonstrable in the aqueous extracts of 4 mammary carcinomata induced by methylcholanthrene in female mice which were susceptible to the development of spontaneous mammary carcinoma but did not possess the milk agent before treatment because of foster-nursing. The action of the carcinogen was thus not dependent upon the presence of a milk agent.

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Experimental Artificial Left Heart to Permit Surgical Exposure of Mitral Valve in Cats.* (17852)

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The most promising among the methods devised to make practicable intracardiac surgery appear to be those which artificially support the circulation and render the heart bloodless for finite periods of time(1-10). The difficulties encountered in these various

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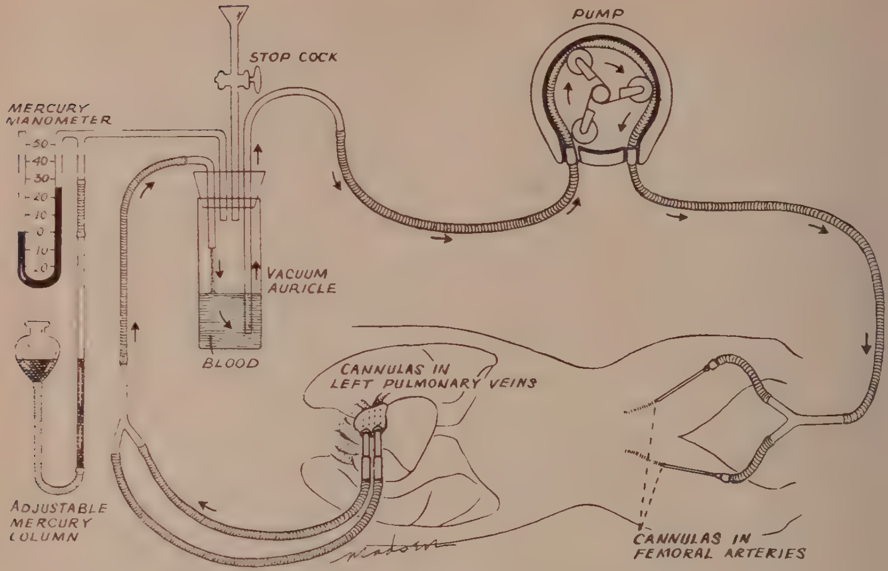
PUMP
STOP COCK
MERCURY MANOMETER
-50-
40
30
20
10
0
10
20
VACUUM AURICLE
BLOOD
CANNULAS IN LEFT PULMONARY VEINS
ADJUSTABLE MERCURY COLUMN
CANNULAS IN FEMORAL ARTERIES

FIG. 1.
Diagram of Extracorporeal Circuit. Arrows indicate direction of blood flow. See text.

bloodpumping and oxygenating systems have generally been in attempting to reproduce the physiology of the lungs. The authors feel that further experimental development of the surgery of the mitral area need not await perfection of oxygenating systems. All that is required for exposure of the mitral valve is that the left side of the heart be bloodless. Our system consists essentially of a method of delivering normally oxygenated blood from the left lung into a glass chamber and thence pumping it into the arterial tree via the femoral arteries; while at the same time, blood from the right lung is prevented from entering the left heart by temporarily occluding the right pulmonary artery with a tourniquet (Fig. 1).

Method. I. *Apparatus.* The apparatus used in this method consists of four main elements (Fig. 2 and 3):

A. *Two stainless steel cannulae* for the left pulmonary veins.

B. *A closed glass chamber* to act as the auricular reservoir. There are 4 openings to this chamber. One opening admits a glass tube which delivers the blood collected from the left lung. A second admits a glass tube to the bottom of the chamber which collects the blood and carries it to the pump. The third admits a glass tube with a stopcock and funnel so that the system may be closed or opened to atmospheric pressure and through which fluids may be added. The fourth admits a glass tube which is connected to an adjustable mercury column and a mercury

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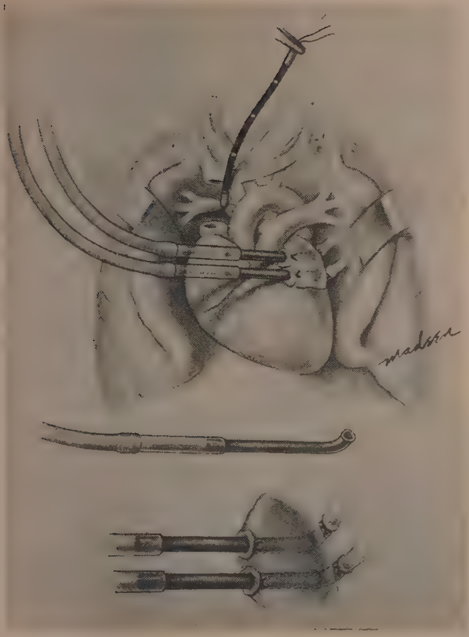

FIG. 2.
(A) Illustration of cannulae in place in left pulmonary veins, and tourniquet occluding right pulmonary artery. (B) Illustration of one pulmonary vein cannula. (C) Cannulae passing through left auricular appendage and auricle and fixed into the left pulmonary veins.

manometer. By controlling the height of the mercury column the pressure in the vacuum auricle can be altered and read on the mercury manometer.

C. *A modified roller-type pump* such as has been used by DeBakey(11) and Gibbon (4). The position of the rollers was controlled by having them ride on completely circular tracks, being held there by phosphor bronze leaf springs. The lumen was controlled by use of shims and thus could be varied precisely by changing the total shim thickness. The creeping of the rubber tube due to the rolling action was prevented by the use of rubber cuffs cemented to the outside of the rubber tube.

D. *Latex rubber tubing* connecting all of the above-described elements. All of the equipment which came in contact with blood was silicone-coated with GE Dri-Film No. 9987.

II. *Technical problems.* Collapse of the pulmonary veins with sealing of the openings of the cannulae by the walls of the veins was encountered. The cannulae, therefore, had to be so designed that they could be aligned with the walls of the vessels; and, the surges of negative pressure, which drew the blood from the veins, and tended to collapse them suddenly, had to be smoothed out. This was done by introducing an air chamber (the artificial auricle) between the cannulae and the pump. It was found that the blood could be removed from the pulmonary veins more rapidly and that the artificial system would be more stable, if the pressure in the artificial auricle was reduced to between –20 and –30 mm of mercury. Other problems arose at the arterial end of the artificial system. In our early attempts the blood was returned to the aorta via a cannula placed in the proximal end of the divided subclavian artery. Seventeen cats survived this procedure for periods varying from 2 to 20 hours, but all

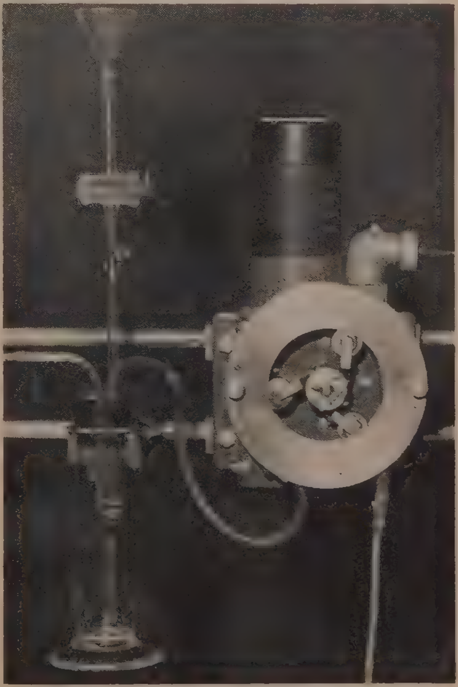

FIG. 3.
Photograph of vacuum auricle and pump.

died without fully regaining consciousness. We believed that this was due to cerebral ischemia rather than insufficient oxygenation of blood because there had been no cyanosis. On reexamining the system, it was realized that large quantities of blood had to be moved into the aorta through the comparatively small orifice of the subclavian artery. A high pressure jet was thus produced which had an aspirating effect on the adjacent opening of the innominate artery and this probably removed significant quantities of blood from the cerebral circulation. This problem was solved by returning the blood to the aorta via cannulae directed cephalad in each femoral artery.

III. *Technic.* The technic used is as follows: The animal is anaesthetized. The right and left femoral arteries are exposed for the insertion of cannulae. The chest is opened by splitting the sternum and both pleural cavities are entered. An artificial respirator attached to an endotracheal tube maintains the respiration with atmospheric air. The right pulmonary artery is dissected free. A silk ligature is passed around it and allowed to lie loosely in place. The left superior and inferior pulmonary veins are dissected free. Elastic threads are passed around them and allowed to lie loosely in place. After heparinization of the animal, cannulae directed cephalad are fixed in both femoral arteries. The pulmonary vein cannulae are then passed through the left auricular appendage and chamber of the left auricle into the openings of the left superior and inferior pulmonary veins. They are fixed in this position with the previously placed elastic threads. The artificial system having been filled with blood from a donor cat is then started. By lowering the mercury column, the pressure in the vacuum auricle is now adjusted to a negative pressure of –25 mm of mercury. The circulation is then maintained for approximately one minute with the animal's left heart and the artificial system in operation simultaneously: the blood coming from the right lung still passes through the left side of the animal's own heart while the blood coming from the left lung is collected by the cannulae and delivered via the artificial system to the aorta. By the end of this time, the previously placed silk ligature tourniquet (Fig. 2A) around the right pulmonary artery is gradually tightened, occluding this vessel. Thus, all of the blood, henceforth, is shunted through the left lung and the artificial system. At this point, either the left auricle or the left ventricle is opened. The steps to discontinue the artificial and restore the normal circulation are now begun. The exploratory incision in the auricle or ventricle is closed. The tourniquet around the right pulmonary artery is gradually released allowing blood to begin passing through the right lung and returning to the animal's left auricle. The elastic ligatures around the cannulae in the pulmonary veins are cut and the cannulae withdrawn from the veins into the animal's left auricular chamber. The pump is stopped and the cannulae removed by withdrawing them until their tips are in the auricular appendage and by ligating the appendage. Protamine sulfate is administered intravenously to counteract the previously administered heparin. The pericardium is loosely reapproximated and the chest wound closed in layers. The cannulae are removed from the femoral arteries and these vessels ligated.

Results. A total of 60 cats were used in this series. The first 53 were lost in the process of developing the final technic. Of the last 7 animals, all recovered from the procedure and survived for periods exceeding 36 hours except for one which was immediately sacrificed to obtain data. Four expired in periods ranging from 36 to 54 hours postoperatively. Two animals which are living to the date of this writing (a period of 3 months) appear to enjoy normal health (Table I). In the last 24 experiments, either the left auricle or the left ventricle was opened while the circulation was maintained by the artificial system and, in every instance, found to be practically bloodless. Direct blood pressure readings from the left subclavian artery revealed that the mean pressure rarely fell below 100 mm of mercury during the entire procedure including the period on the artificial heart.

Discussion. Hemolysis, which might have

TABLE I.
Summary of Results of Experiments Following the Development of the Technic.

Cat No.	Min. on artificial L. heart	Date of exp.	Hr. survived	Autopsy findings
54	28	12/14/49	36	Mucus plug in trachea.
55	15	17	48	Empyema, bilateral, lobar pneumonia.
56	14	19	*	
57	40	20	46	Meningitis.
58	29	24	54	Tracheo-bronchia tree blocked by thick secretions.
59	40	27	0	Sacrificed immediately after procedure. No abnormal findings.
60	32	29	*	

* Alive and well at this writing, a period of 3 mo.

been anticipated due to rapid circulation of blood in large quantities through an artificial system, was not a problem in our technic. By coating the equipment with silicone and designing the pump so that the walls of the rubber tubing never quite collapsed when compressed by the roller, thus minimizing crushing and shearing of red cells, hemolysis became insignificant. Tests for hemolysis in which one unit of citrated human bank blood was circulated through the artificial system at the rapid rate of 500 cc per minute for one hour revealed only faint traces of hemoglobin in the plasma. Although no measurements of the oxygen saturation were made, arterial blood always appeared to be bright red. Experience with pneumonectomies has shown that man and other animals survive indefinitely with one lung removed. It is still to be established whether a human suffering from long-standing mitral stenosis can safely oxygenate all of his blood on one lung for a period long enough to permit intracardiac surgery. It can be seen from Fig. 4 that the blood pressure with the artificial system is essentially a mean pressure, reflecting the surges inherent in the pump whose output is otherwise non-pulsatile. While others(10,12,13) have reported that pulsatile flow is necessary for the normal functioning of organs, it has been our experience that an essentially non-pulsatile flow at approximately normal mean pressures, for periods up to one-half hour, is consistent with the animal's survival.

In this report a method is presented which provides opportunity for further experimental surgery of the mitral valve by rendering the mitral area bloodless for finite periods of time without the use of an artificial oxygenator. Extra-corporeal systems which include oxygenators and also accomplish the above purpose have a broader field of application. Both the right and left hearts, as well as the lungs, are nonfunctioning and bloodless while such an artificial system is in operation. Such surgical problems as the repair of septal defects and operative procedures on the pulmonary arteries are amenable to a direct visual approach with such systems. A further advantage is that the introduction of cannulae to obtain the blood from the superior and inferior vena cavae is technically simpler than the introduction of cannulae into the pulmonary veins. Finally, an artificial heart-lung system might be used as an emergency procedure to tide a patient over a serious cardiopulmonary crisis. On the other hand, it appears that for experimental mitral surgery, systems including oxygenators are more complex than necessary. Moreover, they have not yet reached a stage that allows their ready reproduction and use in laboratories primarily interested in experimental mitral surgery. Our system has only a specialized application in that it would be used for intracardiac surgery of the left heart. For this purpose it seems ade-

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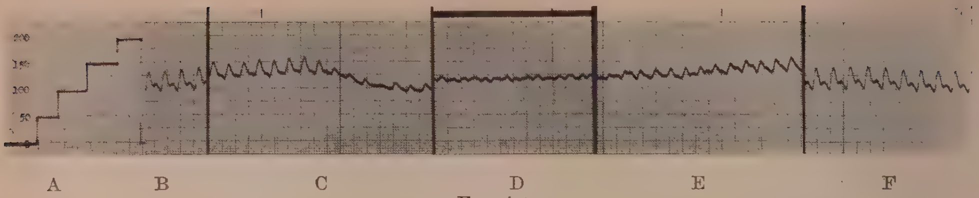

FIG. 4.

Sections of continuous blood pressure tracings in left subclavian artery of Cat No. 59 made with a Sanborn electromanometer and electrocardiograph. (A) Calibration in mm mercury. (B) Before introduction of cannulae. (C) Animal's left heart and artificial system in operation simultaneously. Latter portion: Occlusion of right ~~pulmonary~~ artery with consequent elimination of animal's left heart. (D) Animal completely maintained on artificial system. (E) Change over from artificial system to both animal's left heart and artificial system. (F) Conclusion of experiment.

quate. Its simplicity allows ready reproduction of the equipment in laboratories with modest mechanical facilities. Theoretically, it is conceivable that such a system could be applied to make bloodless the right chambers of the heart§, and even with 2 such systems to make the entire heart bloodless without an oxygenator. The dexterity required to place the cannulae in the pulmonary veins certainly makes less demand upon surgical skill than is generally required in attacking so difficult a problem as intracardiac surgery. The necessity of opening the chest and the heart to introduce the pulmonary vein cannulae is no disadvantage, as this must be done with any system in order to expose the mitral valve. In our system, the blood comes in contact with less artificial equipment and less blood (15 cc in the cat) is required to initially fill the system. Finally, it should be pointed out that the hemodynamics of our system are partly controlled by an intact and functioning right heart which responds to the afferent stimuli from the total animal.

Summary. 1. A system of bypassing the left heart to expose the mitral area for surgery is described.

2. The system consists of delivering normally oxygenated blood from the left lung via cannulae in the pulmonary veins, into a glass chamber and thence, pumping it into the aorta via the femoral arteries, while blood from the right lung is temporarily prevented from entering the left heart. Blood pressures are always maintained at adequate levels, and hemolysis is insignificant.

3. It differs from previously reported extra-corporeal systems in that the right heart and left lung continue to function, eliminating the need for artificially reproducing the physiology of the lungs.

4. In the 7 experiments after the development of the final technic, one cat was immediately sacrificed to obtain data, 4 survived for 36-54 hours and 2 are alive and well at this writing (a period of 3 months).

The authors are grateful for the guidance and help of Dr. H. M. Zimmerman. Dr. Clarence J. Schein assisted at some surgical procedures. Dr. Jacob Halperin aided in recording and interpreting the blood pressure tracings and electrocardiograms. Neuropathological studies were done by Dr. Martin G. Netsky. Mr. Ruthven C. Ferreira rendered valuable technical assistance.

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§ During the progress of this work, Sewell and Glenn reported an experimental method of bypassing the right side of the heart. (Meeting of the American College of Surgeons, Chicago, Oct., 1949.)

Antigenic Similarity of Bovine Strains of *Leptospirae* (United States) and *Leptospira pomona.* (17853)

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(Introduced by Joseph E. Smadel.)

From the Veterinary Division, Army Medical Department, Research and Graduate School, Washington, D.C.

Sera from horses affected with recurrent iridocyclitis contain antibodies against *Leptospira pomona* and the New Jersey C164 strain of bovine leptospira(1). *L. pomona* was originally isolated in 1936 from a dairy farmer in Australia(2) and has since been found in Italy, Savoy, and Switzerland(3), but has not as yet been reported to occur in the United States.

Methods. The strains of leptospira used in these studies were: *L. icterohaemorrhagiae,*[*] *L. canicola,*[†] *L. pomona,*[*] *L. bovis (Baruch Strain),*[‡] New Jersey bovine leptospira strain C164,[*] and New York bovine leptospira strain A.[§] The positive sera employed included one from a patient who acquired a natural infection with *L. pomona* in Malaya, bovine serum from a naturally infected steer in Kansas, and hyperimmune sera produced experimentally in rabbits. Of the latter, one serum, "Lapin 97", hyperimmunized against *L. pomona,* was obtained from the Pasteur Institute in Paris through the courtesy of Mme. Kolochine-Erber. Three to 4-day-old actively growing cultures of leptospirae cultivated in Schüffner's medium incubated at 30°C were used as antigens in microscopic agglutination-lysis tests which

TABLE I.
Cross Agglutination-Lysis Tests.

		Titer							
Serum	Antigen	10	50	100	500	1000	5000	10000	50000
Kansas Steer No. 190	*L. icterohaemorrhagiae*	+	0	0	0	0	0	0	0
	L. canicola	0	0	0	0	0	0	0	0
	L. bovis (Baruch)	0	0	0	0	0	0	0	0
	N.J. C164	+	+	+	+	+	+	±	0
	N.Y. A	+	+	+	+	+	+	0	0
	L. pomona	+	+	+	+	+	+	0	0
Hyperimmune Rabbit L97 Pasteur Inst.	*L. icterohaemorrhagiae*	+	±	0	0	0	0	0	0
	L. canicola	+	0	0	0	0	0	0	0
	L. bovis (Baruch)	0	0	0	0	0	0	0	0
	N.J. C164	+	+	+	+	+	+	+	0
	N.Y. A	+	+	+	+	+	+	+	±
	L. pomona	+	+	+	+	+	+	±	0
Human Patient R, Malaya	*L. icterohaemorrhagiae*	0	0	0	0	0	0	0	0
	L. canicola	0	0	0	0	0	0	0	0
	L. bovis (Baruch)	0	0	0	0	0	0	0	0
	N.J. C164	+	+	+	±	0	0	0	0
	N.Y. A	+	+	+	±	0	0	0	0
	L. pomona	+	+	+	±	0	0	0	0

+, All organisms agglutinated or lysed.
±, Many organisms agglutinated or lysed.
0, few to no organisms agglutinated or lysed.

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[*] Obtained October 1949 from the National Institutes of Health, Bethesda, Md.

[†] Isolated October 1948 at the Army Medical Dept. Research and Graduate School.

[‡] Obtained February 1950 from the Hebrew University, Jerusalem.

[§] Obtained February 1950 from Dr. Karl Reinhard, Cornell University, Ithaca, N. Y.

TABLE II.
Cross Absorption Tests.

Serum of rabbit hyperimmunized against:	Agglutination-lysis test antigen	Titer					
			Absorbed with:				
		Not absorbed	*L. icterohem.*	*L. canicola*	*L. pomona*	New Jersey C164	New York A
L. icterohemorrhagiae	*L. icterohemorrhagiae*	100000	5000	100000	100000	100000	100000
	L. canicola	10000	1000	0	1000	1000	1000
	L. pomona	1000	0	0	0	0	0
	N.J. C164	1000	0	0	0	0	0
	N.Y. A	0	0	0	0	0	0
L. canicola	*L. icterohemorrhagiae*	5000	0	0	1000	0	0
	L. canicola	500000	100000	0	100000	100000	100000
	L. pomona	0	0	0	0	0	0
	N.J. C164	0	0	0	0	0	0
	N.Y. A	0	0	0	0	0	0
L. pomona	*L. icterohemorrhagiae*	0	0	0	0	0	0
	L. canicola	0	0	0	0	0	0
	L. pomona	100000	100000	50000	0	1000	1000
	N.J. C164	100000	50000	50000	0	0	0
	N.Y. A	50000	50000	50000	0	1000	0
N.J. C164	*L. icterohemorrhagiae*	10000	0	10000	0	1000	1000
	L. canicola	0	0	0	0	0	0
	L. pomona	10000	10000	10000	0	1000	0
	N.J. C164	100000	10000	50000	0	0	0
	N.Y. A	100000	10000	10000	0	0	0

were performed by the technic of Schüffner and Mochtar(4). Results were recorded as + when all organisms were agglutinated or lysed; ± when many organisms were agglutinated or lysed; and 0 when few to no organisms were agglutinated or lysed. Immune rabbit sera were absorbed with 5 different strains of leptospirae and retested for agglutinins. The absorption technic of Schüffner and Bohlander(5) was employed with the following modifications: living leptospirae grown in Stuart's medium were collected by centrifugation for 10 minutes at 16,000 rpm, and resuspended in buffered saline solution equal to one tenth the original volume of culture. Each of the immune sera was diluted before absorption so that its agglutination-lysis titer was approximately 1:50,000. In general 3 ml of a concentrated suspension of leptospirae were added to 2 ml of immune sera and the mixtures were incubated for 18 hours at 30°C, after which the leptospirae were removed by high speed centrifugation. Such an absorption procedure was repeated twice more before the sera were tested for residual antibodies.

It has been found in this laboratory that the New York A strain of bovine leptospira is pathogenic for weanling hamsters when injected intraperitoneally and can be maintained readily in this host by passage of heart's blood taken when the animals are moribund on the 4th or 5th day after inoculation. In order to determine the protective value of antisera against artificial infection with this strain of leptospira, a hamster protection test, modeled after the mouse potency test used in evaluating anti-swine-erysipelas serum(6) was employed. Groups of weanling hamsters were injected subcutaneously with 1 ml of immune rabbit serum. Twenty-four hours later, they were inoculated intraperitoneally with 0.5 ml of a virulent culture of New York A strain of bovine leptospira.

Results. Cross agglutination-lysis tests were performed on 3 sera from widely different sources, a naturally infected human being in Malaya, a naturally infected bovine in Kansas, and an experimentally immunized rabbit in France. These sera gave the same titration end points with *L. pomona* as with the American strains of bovine leptospira, but did not react significantly with *L. icterohaemorrhagiae, L. canicola,* or *L. bovis (Baruch Strain).* Results of these cross agglutination-lysis tests are shown in Table I.

TABLE III.
Hamster Protection Test.

Group	Immune serum	Hamsters surviving
1.	*L. icterohaemorrhagiae*	0/4
2.	*L. canicola*	0/4
3.	*L. pomona*	4/4
4.	N.J. C164	4/4
5.	None—control	0/4

Absorption tests clearly indicate that antibodies against *L. icterohaemorrhagiae, L. canicola, L. pomona,* and the New Jersey C164 strain of bovine leptospira were removed by absorption with homologous organisms, and that the first 2 leptospirae listed were unrelated to the last two. However, *L. pomona* and the New Jersey C164 and New York A strains of bovine leptospira were indistinguishable on the basis of cross absorption studies. The results of these tests are shown in Table II.

Hamsters passively immunized by the subcutaneous injection of *L. pomona* immune serum and hamsters similarly immunized with New Jersey C164 bovine leptospira immune serum were protected against infection with the virulent New York A strain of bovine leptospira. No protection was afforded hamsters receiving either *L. icterohaemorrhagiae* immune serum or *L. canicola* immune serum. It is worth mentioning that the initial infecting agent was recovered from each of the animals which succumbed but not from those which survived. The results of this test are shown in Table III.

Summary. The results of agglutination-lysis, absorption, and animal protection tests indicate that the strains of bovine leptospira of American origin designated as New Jersey

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4. Schüffner, W., and Mochtar, A., *Zbl. f. Bakt.,* 1927, Org. 101, 405.

5. Schüffner, W., and Bohlander, H., *Zbl. f. Bakt.,* 1939, Org. 144, 434.

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C164 and New York A are indistinguishable from *L. pomona.* These findings stress the need for reclassification of the American strains. They also indicate that *L. pomona* or an antigenically indistinguishable organism is present in North America.

The authors acknowledge with thanks the technical assistance of Frank H. Smiley, and LaRue B. Evans.

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Protective Effect of Para-Aminopropiophenone Against Lethal Doses of X-Radiation. (17854)

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Yeasts(1), plants(2,3) and both normal tissues and tumors of mammals(4-9) have been protected against radiation injury by reducing the oxygen tension during the period of exposure to radiation. The reduction of oxygen tension has been accomplished in a variety of ways. Tumors have been rendered hypoxic by *in vitro* enclosure in a nitrogen atmosphere during radiation(6), and portions of intact animals have been made hypoxic by occlusion of blood vessels by either pressure or ligation(4,5,8). Hypoxia during the period of irradiation has been produced in the entire animal by strapping the chest to interfere with respiration(8), by bleeding the animal immediately prior to irradiation (7), and by enclosure in a low oxygen, high nitrogen atmosphere(9). These methods are somewhat cumbersome and with one exception the evaluation of the degree of protection afforded has depended on estimations of radiation damage based on gross or histologic observations. In the instance where animals were exposed in a low oxygen atmosphere weight changes and 30-day mortality were used as criteria for the evaluation of protection. Cyanide has been shown to exert some protective action against X-radiation in mice, but this effect was not necessarily attributed to the histotoxic anoxia produced(10). The present study was undertaken to determine whether or not hypoxia produced by other chemical means during the period of exposure to X-radiation would protect intact animals against radiation damage. Para-aminopropiophenone (PAPP),† a compound shown to produce methemoglobinemia of relatively long duration(11), was

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† Obtained from Dow Chemical Co., Midland, Mich.

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TABLE I.
30-Day Survival in Control and Para-aminopropiophenone (PAPP) Treated Mice Following 800 r Total-Body X-ray.

Dose of PAPP intraperitoneally mg/kg	% methemoglobin 30′ after injection (Avg for 4 mice)	Time of injection of PAPP	30-day survival	% survival
20	55	30′ before X-ray	9/20	45
30	59	30′ ,, ,,	10/20	50
40	65	30′ ,, ,,	12/20	60
50	75	30′ ,, ,,	29/40	72.5
50	—	10′ after ,,	0/20	0
0	—	—	0/60	0

chosen as the chemical agent for producing hypoxia.

Materials and methods. A total of 180 female CF_1 mice, weighing 21-26 g, were given 800 roentgens of X-ray in a single total-body exposure (250 kv, 15 ma, ¼ mm Cu, 1 mm Al, 50 cm distance, and 35-40 r/min). Control and experimental animals were irradiated simultaneously in groups of 20. Each mouse was enclosed in an individual compartment of a cylindrical cage, each compartment being equi-distant from the center of the field, and the cage rotated continuously during the exposure period. Preliminary toxicity studies showed the LD_{50} for mice injected intraperitoneally with a 1% solution of PAPP in propylene glycol to be approximately 65 mg/kg. Doses of 20, 30, 40, and 50 mg/kg, which killed no animals in the toxicity studies, were chosen to test for prophylactic effect against radiation damage. A total of 100 mice received these dosages 30 minutes before exposure to radiation. Twenty mice received 50 mg/kg 10 minutes after exposure and 60 were irradiated as controls.

Blood methemoglobin levels for each dose of PAPP were determined by a modification of the method of Evelyn and Malloy(12). One-tenth ml of blood was removed by cardiac puncture and laked in 5 ml M/20 phosphate buffer, pH 7.4, containing 0.1% saponin. The sample was then centrifuged for 3 to 5 minutes and samples were poured into each of 2 cuvettes. A drop of 20% potassium ferricyanide was added to the second tube. Extinctions were then read in a Coleman Spectrophotometer at 630 mu. A drop of 2% sodium cyanide was added to both tubes and the extinctions were again read. The percentage of methemoglobin was then calculated by the following formula:

$$\frac{\text{Drop in extinction of first tube}}{\text{Drop in extinction of second tube (ferricyanide)}} \times 100 = \text{\% methemoglobin in sample}$$

Results. Determination of methemoglobin levels in mice following the intraperitoneal injection of PAPP showed that maximum levels were reached in about 30 minutes with a subsequent slow drop in the level. At 60 minutes the level was never more than 10% lower than the 30 minute value. Since the mice were irradiated during this interval the exposure took place when methemoglobin levels were at or near the maximum.

The mortality data are summarized in Table I. Eight hundred roentgens killed 100% of the control mice. The presence of high levels of methemoglobinemia during the time of irradiation exerted an appreciable protective action. Levels of approximately 75% methemoglobin reduced the mortality from 100% to less than 30%. Lower levels of methemoglobin produced less profound but appreciable protection. Administration of PAPP 5 minutes after irradiation was without effect on the 30-day mortality or death time. At the higher doses of PAPP the mice became extremely cyanotic and prostrated but could be aroused by stimulation. These doses did not produce any mortality *per se*.

A similar test on Sprague-Dawley rats given 30 mg/kg of PAPP 30 minutes before exposure to 800 r total body X-ray showed

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12. Evelyn, K. A., and Malloy, H. T., *J. Biol. Chem.*, 1938, v126, 655.

75% survival in 12 treated and 10% survival in 20 control rats 30 days after irradiation.

Discussion. It seems logical to suppose that PAPP exerts its prophylaxis against the lethal effects of X-ray by the hypoxia resulting from the severe and relatively prolonged methemoglobinemia. The possibility, however, that PAPP may exert its prophylactic effect by some mechanism other than the production of methemoglobinemia is being investigated by testing other unrelated compounds which are known to produce methemoglobinemia. Preliminary studies on sodium nitrite have shown that a dose of 100 mg/kg given intraperitoneally 10 minutes before irradiation with 800 r is without effect on subsequent mortality. This has been confirmed by other workers(13). Determination of the degree of methemoglobinemia produced by this dose of sodium nitrite showed an average of 52% for 4 mice 10 minutes after injection. By 30 minutes, the end of the X-ray exposure period, the level had fallen to 46%. While this level is slightly less than that produced by the lowest dose of PAPP tested, it does not seem likely that this would explain the marked difference in results. The reason for this inconsistency is being investigated further. The use of PAPP to produce hypoxia offers several distinct advantages over previous methods. No cumbersome procedures are needed, the extent of methemoglobinemia and the degree of hypoxia can be easily controlled by varying the dose of PAPP, and considerably less than lethal doses produce the desired results. Doses as low as 20 or 30 mg/kg shifted the 30-day LD_{50} from approximately 515 r(14) to about 800 r of X-radiation. Higher doses of PAPP produced an even more pronounced shift.

Summary. Para-aminopropiophenone (PAPP) when injected intraperitoneally in mice produces a maximum methemoglobinemia in 30 minutes with only a slight decrease in the methemoglobin level after 60 minutes. Of 180 mice given a single exposure of 800 r total-body X-radiation 100% of 60 controls died within 30 days. Pre-treatment of groups of the remaining 120 mice with PAPP in doses of 20, 30, 40, and 50 mg/kg, 30 minutes before X-ray exposure, resulted in 30-day survival rates of 45, 50, 60, and 72.5%, respectively. Injection of 50 mg/kg of PAPP after irradiation did not affect mortality. In a preliminary test pre-treatment with PAPP also reduced the 30-day mortality in rats exposed to X-radiation.

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Effect of 4-Amino-Pteroylglutamic Acid (Aminopterin) on Early Pregnancy.* (17855)

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Recent studies have shown that lesions which are characteristic of severe folic acid deficiency can be rapidly induced in mammals by administration of the potent antagonist, 4-amino-pteroylglutamic acid (4-amino-PGA)(1,2). In work with dogs, a

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† Post-doctorate fellow of the National Cancer Institute.

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TABLE I.
The Effect of 4-Amino-PGA on Pregnancies of Rats.

Group	No. of rats	Dosage, mg/kg	Days after mating		Rats with litters	No. of implantations	No. of feti, natural	Damaged	Disintegrated	Reabsorbed
			Injected	Sacrificed						
A	23			14	17	180	180			
B	20	3 × .1	5,6,7	8	3	19		14	5	
C	10	.1	5,6,7	14	5	39		10	5	24
D	21	.15	7,7,8	14	17	130		29	51	50
E	15	.15	7,7,8	23		1				all

bitch, sacrificed several days after treatment with the antagonist, was found to have a dead and macerated litter *in utero*. The observation stimulated further studies of possible effects of 4-amino-PGA on fetal development.

Procedure. In the following experiments Sprague-Dawley and Wistar rats and CFW mice were used. The animals were mature, had previously littered, and weighed between 200 and 250 g (rats) and over 25 g (mice). The animals were separated in individual cages, when pregnant. Their weight was recorded daily while under treatment. Animals were sacrificed, while under ether anesthesia, to avoid artefacts. Uteri were inspected *in situ*, placental implantations counted and measured, and abnormal conditions recorded. Uterine segments with placenta or areas of increased vascular supply were embedded in paraffin, sectioned and stained with Harris hematoxylin and eosin. The sternum of all sacrificed animals was fixed in Vandegrift's and sectioned. Fresh solutions of 4-amino-PGA[‡] were prepared just prior to their intraperitoneal injection. Three consecutive doses of 0.15 mg/kg in mice and 0.1 or 0.15 mg/kg in rats were given at intervals of either 12 or 24 hours.

Exp. I. Twenty-six female mice and 39 female rats (Sprague-Dawley) were mated for a week. The females were then separated from the males. Seven days later the animals were started on a course of 3 daily injections of 4-amino-PGA. They were then permitted to litter. According to the time of mating a week's range of littering was expected. However, counting from the time of the first injection of the drug, all mice born were delivered between the eighth and tenth days, and all rats, between the tenth and twelfth day. Thus, litters were produced only by animals which had been pregnant 8 or more days at the time of the first injection and it appeared that pregnancies younger than 8 days were not maintained. In a second series of 30 female rats (Sprague-Dawley) treated in the same way, only 14 animals littered between 10 and 12 days after the first injection with 4-amino-PGA. However, 14 of 16 control animals, which had been mated for 7 days, littered over a period of one week.

Exp. II. In order to study the effect of the drug during the first week of pregnancy in rats, experiments were carried out with more accurate knowledge of conception time. For this purpose Wistar female rats were permitted to litter. Immediately thereafter, the newly born were removed and the mother remated for 48 hours. A high percentage of conception was anticipated from the ovulation which usually follows within a short time after delivery. Indeed, of 23 control rats sacrificed on the fourteenth day after the beginning of such mating 17 were found to be pregnant with a total of 180 feti (group A, Table I).

In a series of 30 experimental rats, mated as above and injected with the drug on the fifth, sixth, and seventh day following the previous littering, only 8 showed evidence of pregnancy with 58 sites of implantation (groups B and C, Table I). The uterine notches in the treated rats were only 3 to 4

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[‡] The authors are indebted to the Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y., for supplies of 4-amino-PGA.

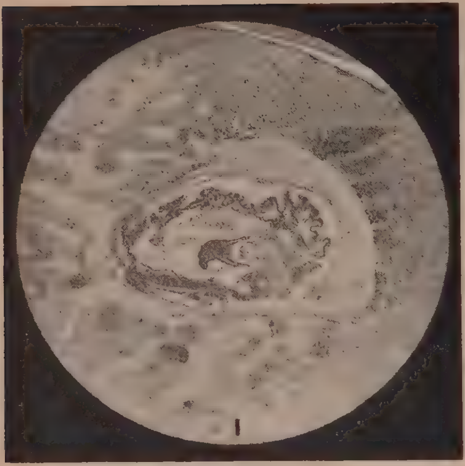
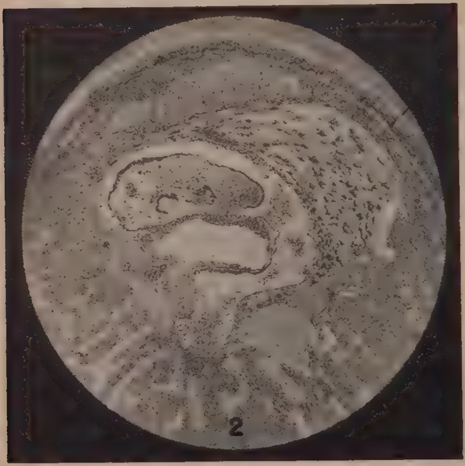


FIG. 1.
Control 8-day rat embryo.



FIGS. 2, 3, 4.
Rat embryos 14 days old; mothers injected 3 times with 0.15 mg/kg of 4-amino-PGA on 7th, 7th, and 8th day of pregnancy.

FIG. 2. Intact amnion surrounds damaged fetus. Organ structures still recognizable, mesenchyme damaged.

mm in diameter irrespective whether the animals were sacrificed at 8 or 14 days. The notches of control rats (Group A) sacrificed on the fourteenth day, were 1 cm in diameter. Two uteri of the experimental group were filled with a dark bloody fluid between the hemorrhagic implantation sites.

Of a further series of 21 rats, injected on the seventh and eighth day after the previous littering, 17 showed evidence of pregnancy when sacrificed with 130 sites of implantation (Group D, Table I). Again the uterine notches were approximately 4 mm in diameter and no whole feti could be found. Two animals of group D had distended uteri filled with a thick bloody fluid between 4 sites of implantation.

A further series of 15 rats injected on the 7th, 7th and 8th day after the previous littering (group E, Table I) and sacrificed

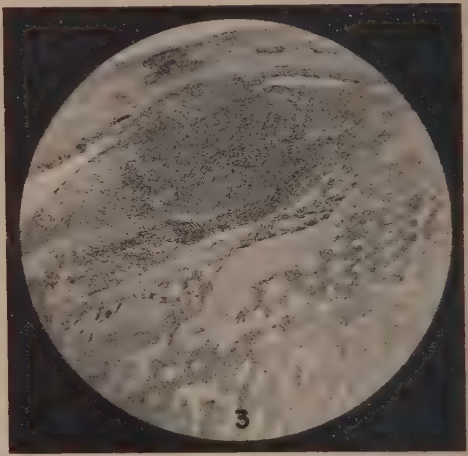


FIG. 3. Necrotic fetus infiltrated with leucocytes.

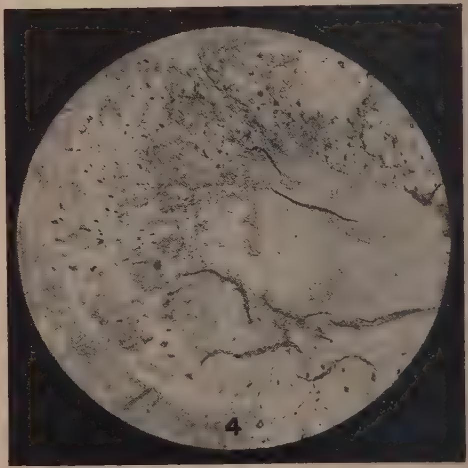


FIG. 4. Necrotic fetus resorbed, central area filled with blood. Early involution of placenta.

after 23 days had no litters at all. In only one rat one previous site of implantation was found consisting of a small blood filled notch in the uterus 3 mm in diameter.

Microscopical examination. Previous studies had shown 4-amino-PGA to have a depressing effect on the bone marrow(1). The same lesion was found in the present animals. When examined 24 hours after the last injection of 4-amino-PGA, the bone marrow was depleted approximately 50 per cent with respect to myelo- and erythropoietic elements. Within one week after the last injection, the marrow was completely recovered and showed normal cellularity.

No lesions were found in the muscular wall of the uterus in any experimental animal. The rats of group B (Table I), which contained litters, showed decidual reaction of the endometrium with early implantation of small placentas. These implantations filled the lumen of the uterus like small plugs. Deciduas and placentas appeared to be intact but the feti were damaged. The internal organs of the embryos did not stain well and many nuclei had lost good definition.

Rats sacrificed on the fourteenth day of pregnancy and 7 days after the last 4-amino-PGA injection (groups C and D, Table I) provided better evidence of fetal damage and death than did the previous group. Again no lesions were found in the muscular coat of the uterus. The placentas corresponded to those of 7-day embryos and were intact in most cases. In 22 animals evidence of pregnancy existed in the presence of 169 sites of implantation. Undamaged feti, however, were not found. Thirty-nine of these implantations contained dead feti in which organ structure could still be recognized but in which most of the mesenchymal elements had degenerated and disintegrated. The embryos were surrounded in most cases by an intact amnion. In 56 instances the feti had shrunken to a mass of debris which formed a thin thread infiltrated with leucocytes and surrounded by placentas. In the remaining 74 implantations no fetal tissue was detected and it appeared that the embryonic debris had either been resorbed or emptied into the uterine lumen. The latter process was suspected in those cases in which the lumen was filled with blood and adjacent placentas and deciduas were undergoing involution.

Rats sacrificed 23 days after mating (group E) showed complete resorption of feti and placentas. Only in one instance a small placental rest with a central hemorrhage was detected.

Discussion. The findings above indicate that the doses of 4-amino-PGA used were toxic to pregnant rats insofar as they caused a temporary depletion of bone marrow. The treated animals lost a small amount of weight but did not become seriously ill. However, their embryos were irreversibly damaged when treatment was initiated at time of implantation. Histological study indicated the site of action of the drug to be in the embryonic mesenchyme. Decidual and placental tissue appeared to be normal in most cases. From this one would assume a direct toxic action of the compound on the fetus. Embryos of rats and mice older than 10 days proved more resistant to the antivitamin in preliminary experiments. This observation agrees with studies on the chick embryo in which a direct relationship was demonstrated between age of embryo and amount of 4-amino-PGA necessary to effect death(3). The results of the present experiments confirm the observation of Nelson and Evans who studied reproduction in female rats kept on folic acid deficient diets supplemented with crude "x-methyl-folic acid"(4). These authors found complete resorption of litters when the crude antagonist of folic acid was fed from the first day of mating. The experiments described above, however, demonstrate that the potent antagonist, 4-amino-PGA, can destroy a well-established pregnancy when the agent is given in only 3 small doses. If one accepts the hypothesis of the antifolic acid action of 4-amino-PGA, one

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4. Nelson, M. N., and Evans, H. M., *J. Nutrition*, 1949, v38, 11.

has to conclude that folic acid is necessary for the maintenance of early fetal life in mice and rats. This conclusion is also advanced by Nelson and Evans(4).

Summary. 4-amino-pteroylglutamic acid was found to be lethal to the early embryo of mice and rats. Fetal death was observed with doses which temporarily caused depletion of the bone marrow but no other significant signs of intoxication in mothers.

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Partial Purification of Viruses with an Anion Exchange Resin.* (17856)

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A variety of techniques have been developed for the purification of viruses. Methods which have been applied to poliomyelitis viruses include: adsorption to, and elution from alumina gel(1) or aluminum hydroxide(2); half saturation with ammonium sulfate(3); filtration(4); procedures involving ether extraction, protein precipitation and centrifugation or ultracentrifugation (5-9); methanol precipitation(10); and protein precipitation with protamine sulfate(11). A relatively simple and rapid procedure for the partial purification of the Lansing strain of poliomyelitis virus has been developed in this laboratory. It utilizes a strong-base anion exchange resin. The method is also applicable to the extraction of poliomyelitis virus from human feces and Theiler virus (TO) from mouse feces and presumably may be applied to other viruses as well.

With this method, it is possible to eliminate rapidly the bulk of extraneous material and to obtain a preparation that has not lost a significant amount of virus. Advantages of the method include a considerable saving of time, the requirement of only the simplest equipment, and the elimination of lengthy chemical procedures.

Materials and methods. The Lansing strain of poliomyelitis virus from nervous tissue of mice, poliomyelitis virus from human feces, and Theiler virus obtained from the feces of normal mice were used in these experiments. The Lansing virus and Theiler virus were assayed in 4-5 and 3-4-week-old mice respectively (CFW-albino agouti strain, Carworth Farms), using the intracerebral route of infection. Poliomyelitis virus extracted from human stools was tested intracerebrally in monkeys (*Macaca mulatta*). Of the 3 strong-base anion exchange resins tested (Amberlite XE-56, Amberlite XE-75, and Amberlite XE-67)† only Amberlite XE-67 proved satisfactory for virus purification.

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† Supplied by the courtesy of Rohm & Haas Co., Philadephia, Pa.

The resin is used in the chloride exchange form as it is supplied by the manufacturer. It is a very fine powder, the particles passing through a 325 mesh screen. The following procedure with Amberlite XE-67, carried out at room temperature, proved to be the simplest and most rapid. It is adaptable to the handling of large quantities of infected material and can, if necessary, be carried out without the use of the centrifuge.

(A) Ten grams of dry Amberlite XE-67‡ are added to each 100 ml of a 1% crude suspension of central nervous system tissue (CNS) of mice infected with Lansing virus. The mixture is gently shaken by hand in a stoppered bottle for 20 minutes and then passed through a Buchner funnel using a No. 1 quantitative filter paper. The *effluent* is clear, colorless and non-infective.

(B) The sediment, consisting of a resin-tissue-virus mixture, is washed with sterile distilled water in an amount equal to the original volume; the mixture being stirred in the Buchner cup as it is filtered. This removes the larger particles which have not been adsorbed to the resin. There may or may not be virus in the *wash*.

(C) The resin-tissue-virus sediment is again shaken for 20 minutes in a stoppered bottle with an amount of 10% disodium acid phosphate solution equal to the original volume. This is then passed through a Buchner funnel. The *eluate* is clear, colorless and contains virus. When the eluate is to be inoculated intracerebrally, the excess disodium acid phosphate is first removed by dialysis.

When this procedure is employed to extract human poliomyelitis virus or Theiler virus from feces, the supernatant fluid from a centrifuged 10% fecal suspension is used. Approximately 20% resin by weight is necessary to adsorb the virus from feces. To prevent bacterial infection, 1 mg of Streptomycin and 1000 units of penicillin are added for each milliliter of the final eluate. The procedure is otherwise identical to that described for Lansing virus. The final solution is clear, colorless and relatively non-toxic. It has lost little, if any, of the infectivity of the original suspension. It should be noted that Amberlite XE-67, because of its microscopic particle size, will pass through filter paper if it remains in suspension. It is, therefore, necessary to have a sufficient amount of lipoid or colloidal material in the starting suspension to aggregate the fine Amberlite particles and to retain them in the sediment. For this reason, a 1% crude suspension of nervous tissue prepared in a Waring Blendor is more satisfactory than the supernatant fluid from a highly centrifuged 10% suspension. When fecal material is used, a 10% fecal suspension centrifuged between 2000 and 4000 rpm for 60 minutes at 40°F has been found satisfactory. If however, the effluent is still not clear, centrifugation at 18,000 rpm for 1 hour at 40°F (New International Centrifuge, angle head) is necessary after each step in the procedure. If centrifugation is necessary in the last step, it should be done after the excess disodium acid phosphate has been dialyzed. A modification of the Conway procedure was used to determine total nitrogen content(12,13).

Results. This report is based on 7 experiments with Lansing virus in CNS tissue and 5 experiments with Theiler virus in mouse feces. The examples shown in Table I are representative of each group.

That a considerable amount of extraneous material is removed by the procedure is indicated by the clear, colorless solutions obtained and by the marked decrease in nitrogenous material shown in Table I. The total nitrogen in experiments (b) and (c) is reduced from 288 and 322 γ per ml in the original CNS suspension to 4.2 and 11.2 γ respectively in the final eluate. This represents only 1.5% and 3.5% of the original nitrogen. Similarly, the total nitrogen content of the 10% fecal suspension is reduced

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‡ Although column operations are most often used in exchange-resin work, this procedure is not suitable for Amberlite XE-67 due to its small particle size.

12. Conway, E. J., *Microdiffusion Analysis and Volumetric Error*, p75, D. Van Nostrand Co., New York 1940.

13. Luschinsky, H. L., and Singher, H. O., *Arch. Biochem.*, 1948, v19, 96.

TABLE I.
Partial Purification of Viruses with Anion Exchange Resin (Amberlite XE-67).

Suspensions tested	Exp.	Total N, γ/ml	Infectivity				
			Dilutions				LD$_{50}$ Log 10
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	
			A. Lansing poliomyelitis virus from mouse CNS.				
Original 1% suspension	a	—		4/7*	0/8	0/8	2.0
	b	288		7/7	5/7	0/8	3.3
	c	322		7/7	4/8	2/8	3.2
Effluent	a	—		0/8	—	—	—
	b	9.5		0/9	—	—	—
	c	8.4		0/10	—	—	—
Water wash	a	—		0/8	—	—	—
	b	<1.5		3/8	—	—	—
	c	5.6		4/10	—	—	—
Dialyzed eluate	a	—		4/8	0/7	0/8	2.0
	b	4.2		5/7	3/7	0/8	2.6
	c	11.2		10/10	—	—	—
			B. Theiler virus (TO) from the feces of normal mice.				
Supernatant fluid of a 10% fecal suspension		189	7/8	4/8	0/8	—	1.9
Effluent		68.9	0/8	—	—	—	—
Water wash		35	0/8	—	—	—	—
Dialyzed eluate		18.2	4/6	1/7	0/8	—	1.3

* Numerators indicate the No. of mice paralyzed and denominators No. of mice injected. Mice dying without symptoms of paralysis are omitted.

TABLE II.
Selective Elution of Lansing Poliomyelitis Virus from Amberlite XE-67 with High Concentration of Disodium Acid Phosphate.

Material tested	pH eluate	Infectivity of suspensions
Original 1% CNS suspension	6.6	8/8*
Effluent	5.2	0/8
Eluate with 5% NaCl†	4.9	0/8
" " 10% NaCl	4.8	0/10
" " 5% $NaHCO_3$	9.0	0/10
" " 10% "	9.1	0/10
" " 5% Na_2HPO_4	6.8	0/10
" " 10% "	7.2	6/9

* Numerators indicate the No. of mice paralyzed and denominators No. of mice injected. Mice dying without symptoms of paralysis are omitted.

† Excess salt removed by dialysis before intracerebral inoculation.

from 189 to 18.2 γ of nitrogen per ml. This represents 9.6% of the original nitrogen. It may also be seen in Table I that the final preparations are almost as infective as the original suspensions. The titer in the eluate for the Lansing virus is 2.6 (LD$_{50}$ log of dilution) as compared with a titer of 3.3 in the starting suspension of experiment (b), and the titer in the eluate for the TO virus is 1.3 (LD$_{50}$ log of dilution) as compared with a titer of 1.9 in the starting suspension.

That the virus is adsorbed to the resin is indicated by the infectivity of the various suspensions shown in Table II. The effluent from the resin-tissue-virus complex is non-infective indicating that the virus is retained with the resin. Before attempting to elute the virus from the resin, the complex was thoroughly washed with distilled water to remove any tissue fragments which might contain virus. The wash fluid was occasionally infective (Table I). Aliquots of the resin-tissue-virus complex were then treated with solutions containing different salts at high concentration and at varying pH. Five and 10% solutions of sodium chloride and sodium bicarbonate, and a 5% solution of disodium acid phosphate did not elute the virus from the resin. However, the virus was eluted with a 10% solution of disodium acid phosphate. It appears that the virus is almost selectively eluted with a high concentration of disodium acid phosphate, leaving the bulk of the nitrogenous material adsorbed to the resin (Table I).

Similar results were obtained in the extraction of poliomyelitis virus from pooled human stools of known infectivity. The final eluate which was clear and colorless contained 9.2 γ of total nitrogen as compared with 236 γ of total nitrogen in the starting suspension. The infectivity of the material was retained as shown by the development of paralysis in 1 of 2 monkeys 7 days after intracerebral injection of 1 ml of the final eluate.

Comment. Muller(14) has recently reported the use of a *cation* exchange resin for partial purification of such neurotropic viruses as the Japanese B encephalitis, Eastern equine encephalomyelitis and Western equine encephalomyelitis. The mode of action of Muller's cation exchange resin in effecting a partial purification of virus differs from that of the anion exchange resin reported in this paper. With the use of the *cation* exchange resin, extraneous nitrogenous material is adsorbed to the resin, leaving the virus in the effluent. On the other hand, with the use of the *anion* exchange resin, it is possible to adsorb viruses to the resin along with the nitrogenous material. The virus is then selectively eluted from the resin.

Summary. A simple and rapid procedure for the partial purification of certain viruses is afforded by the use of a strong-base anion exchange resin (Amberlite XE-67). A clear, colorless solution is obtained which retains its original infectivity but which has lost at least 90% of its nitrogenous material.

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14. Muller, H. R., Proc. Soc. Exp. Biol. and Med., 1950, v73, 239.

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Anticonvulsant Action of Isopropyl Alcohol.* (17857)

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In previous reports from this department (1,2), it has been shown that isopropyl alcohol can significantly increase the blood acetone and the cortical threshold to electrical stimulation in different species. Since the maximum acetonemia after gastric administration of the isopropyl alcohol did not strictly coincide with the peak of cortical threshold, further examination of the possible mechanism of the anticonvulsant action has been made.

Methods. The cortical threshold was de-

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* Supported, in part, by the Stern Fund for Research in Anticonvulsants.

1. Driver, R. L., Proc. Soc. Exp. Biol. and Med., 1947, v64, 248.

2. Chu, N., Driver, R. L., and Hanzlik, P. J., *J. Pharm. Exp. Therap.*, 1948, v92, 291.

termined according to the method described by Tainter and associates(3). With increasing amperage, current was passed through the brain for 2.0 seconds at 5-minute intervals until a tonic-clonic convulsive seizure was elicited. Blood acetone was determined by the micromethod of Greenberg and Lester (4). Blood isopropyl alcohol was estimated by a modification of the vacuum distillation, iodometric procedure for ethyl alcohol described by Newman(5).

Gastric administration of isopropyl alcohol. Twenty-two rats and 6 rabbits were each given gastrically the optimal single dose of isopropyl alcohol (1250 mg/kg). Higher doses produce ataxia. Determinations of blood alcohol and acetone and cortical threshold were made 1 hour before and at intervals of 0.5, 1, 3, and 6 hours after the medication. The average results are presented in Fig. 1 and 2. Throughout the period of observation, the cortical threshold in both species rose and fell in a curve approximately parallel to the curve for the blood isopropyl alcohol; while the blood acetone had not reached a peak long after the maximum cortical depression had been achieved.

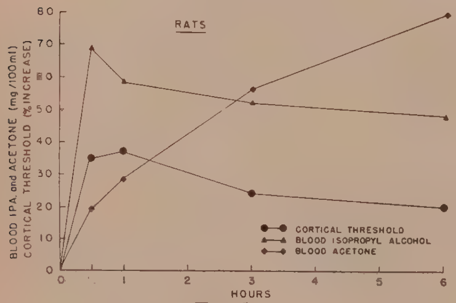

FIG. 1.

Cortical threshold, blood alcohol, and blood acetone following a single gastric dose of isopropyl alcohol, 1250 mg/kg. Averages of 22 rats.

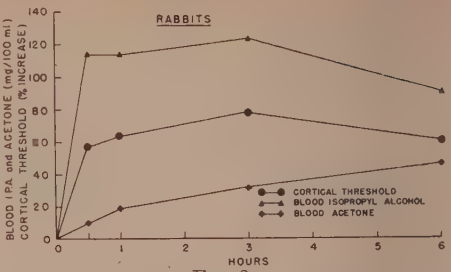

FIG. 2.

Cortical threshold, blood alcohol, and blood acetone following a single gastric dose of isopropyl alcohol, 1250 mg/kg. Averages of 6 rabbits.

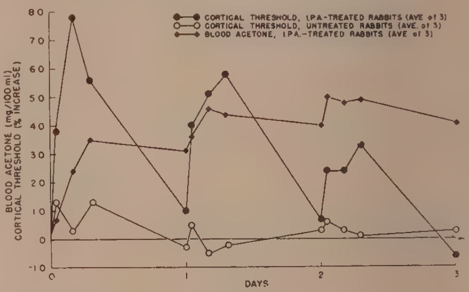

FIG. 3.

Cortical threshold and blood acetone of rabbits which were given isopropyl alcohol gastrically, 1250 mg/kg in 3 equally divided doses daily.

Repeated gastric administration of isopropyl alcohol. Three rabbits were each given 1250 mg/kg gastrically, in equally divided doses, 3 times daily for 3 days. Determinations of cortical threshold and blood acetone were made every morning before, and 1 hour after, each dose. The results are presented in Fig. 3 with the course of cortical thresholds in 3 unmedicated rabbits. Despite a cumulative increase in blood acetone, the daily peaks in cortical threshold showed a diminishing magnitude and each day returned nearly to a zero value. These results confirmed a lack of correlation between cortical depression and blood concentration of acetone.

Intravenous injections of acetone and of isopropyl alcohol. In an attempt to quantitate the share contributed by the acetone and the alcohol in elevating the cortical threshold, these agents were injected intravenously in different groups of rats to produce graded blood levels. Determinations of the cortical thresholds were made 1 hour before, and im-

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4. Greenberg, L. A., and Lester, D., *J. Biol. Chem.*, 1944, v154, 177.

5. Newman, H., *J. Pharm. Exp. Therap.*, 1936, v56, 278.

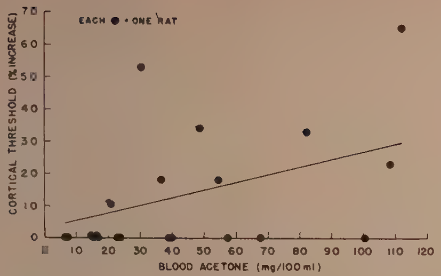

Fig. 4.

Intravenous administration of acetone in rats. (Y = a + bX).

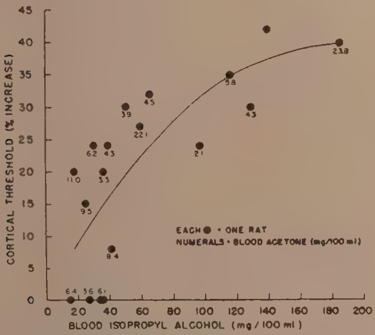

Fig. 5.

Intravenous administration of isopropyl alcohol in rats. (Y = a + bX + cX²).

mediately after, injection. The per cent increase in threshold was plotted against the blood levels of acetone and of isopropyl alcohol (Fig. 4 and 5).

Fig. 4 shows there was a great scatter of values after the intravenous acetone. When the blood acetone was less than 30 mg/100 ml, there was no significant increase in the cortical threshold. Changes of 10% were within the range of experimental error. Over the range of 30 to 110 mg of acetone/100 ml, half of the rats convulsed at the control threshold value, and half had variable threshold increases (18 to 65%). Thus, even at extremely high concentrations of blood acetone, there was no consistent depression of the cortex to electrical stimulation. When isopropyl alcohol was injected, there was less scatter of values (Fig. 5). With blood alcohol levels less than 20 mg/100 ml, there was no significant elevation of cortical threshold; between 20 and 40 mg/100 ml, 4 rats showed no increment in threshold, but 5 rats had elevations of 15 to 24%. In the range of 40 to 183 mg/100 ml, all thresholds were raised significantly (24 to 42%). As higher blood alcohol levels (*ca* 120 mg/100 ml) were reached, there was leveling of the curve, which suggested that the maximum depressant effect had been reached. Since blood samples were drawn within 60 seconds after injection, very little of the isopropyl alcohol had been oxidized to acetone; and the corresponding blood acetone concentrations were low, usually only 1 to 5 mg/100 ml above normal. On the basis of the observations with injected acetone, none of the acetone values in this experiment was great enough *per se* to reduce appreciably the susceptibility of the cortex to electrical stimulation.

Other alcohols. Since these experiments suggested that the anticonvulsant effect of isopropyl alcohol was produced by the alcohol itself, it was of interest to test other alcohols. The results with 4 different alcohols given gastrically to rats are summarized in Table I.

Ethyl alcohol, which yields acetaldehyde rather than acetone, elevated the cortical threshold only after very high doses (7.5 to 10.0 g/kg), which caused definite to marked motor depression. The secondary alcohols, 2-butanol and 3-pentanol, at lower optimum doses (0.5 to 0.75 g/kg, respectively) compared favorably with isopropyl alcohol in raising the cortical threshold but the highest dose caused marked ataxia. 3-Pentanol (0.5 and 0.75 g/kg) yielded comparatively minor increases in blood acetone. The tertiary alcohol, 2-methyl-2-butanol, 0.5 g/kg, produced marked cortical and motor depression without increase in blood acetone.

Chemical convulsants. Tests for isopropyl alcohol antagonism of chemically-induced convulsions were made against the following agents: metrazol, picrotoxin, and thujone (cedar leaf oil). For comparison, similar tests were made with the anticonvulsants, phenobarbital sodium and diphenylhydantoin sodium. For control, the convulsants were given in different doses to groups of 20 rats each. Metrazol, 10%, and picrotoxin, 0.3%, were administered subcutaneously. Oil of

TABLE I.
Various Alcohols on Cortical Threshold, Alcoholemia, Acetonemia and Motor Depression.

Alcohol	Gastric dose (g/kg)	No. of rats	Cortical threshold (% incr.) Avg	Cortical threshold (% incr.) Range	Avg incr. in blood acetone (mg/100 ml)	Avg incr. in blood alcohol (mg/100 ml) Avg	Avg incr. in blood alcohol (mg/100 ml) Range	Motor depression
				Primary				
Ethyl alcohol	2.5	5	0	0				0
	5.0	5	8	0– 38		74	34–132	0
	7.5	5	14	0– 55		100	80–108	Present
	8.5	5	52	0–130				Marked
	10.0	5	45	0– 81				,,
				Secondary				
2-butanol*	.5	5	46	33– 60	18	15	5– 27	0
	.75	8	40	23– 57	24	69	17– 96	0
	1.0	5	85	54–128	31			Marked
3-pentanol†	.5	5	28	8– 43	6			0
	.75	3	33	22– 82	5			Marked
				Tertiary				
2-methyl-2-butanol‡	.5	5	67	40–112	0			,,

* or secondary butyl alcohol.
† or diethyl carbinol.
‡ or tertiary amyl alcohol.

TABLE II.
Isopropyl Alcohol and Other Anticonvulsants Against Chemical Convulsants.
20 rats in each experiment.

Anticonvulsant*	Total convulsions No.†	P	Max. convulsions No.‡	P	Total convulsions No.†	P	Max. convulsions No.‡	P§
				Metrazol.				
	35 mg/kg				50 mg/kg			
None	7		3		20		15	
Isopropyl Alcohol	0	.01	0	.32	15	.01	2	.0009
Diphenylhydantoin	5	.30	4	.39	17	.32	16	.39
Phenobarbital	0	.01	0	.32	8	.0005	7	.08
				Picrotoxin.				
	2 mg/kg				3 mg/kg			
None	19		16		20		20	
Isopropyl Alcohol	15	.39	6	.004	19	.39	17	.16
Diphenylhydantoin	7	.0005	6	.004	16	.12	16	.12
Phenobarbital	6	.0005	4	.0005	14	.02	10	.0005
				Thujone.				
	14 mg/kg				16 mg/kg			
None	10		10		19		17	
Isopropyl Alcohol	9	.39	3	.04	10	.004	4	.0005
Diphenylhydantoin	17	.04	17	.04	16	.32	15	.39
Phenobarbital	8	.39	7	.39	16	.32	8	.008

* The doses of the anticonvulsants throughout were as follows: isopropyl alcohol, 1250 mg/kg; diphenylhydantoin, 100 mg/kg; phenobarbital, 50 mg/kg.
† Number of rats showing min. and max. clonic seizures.
‡ Number of rats showing maximum convulsons.
§ When P is less than 0.05 the difference is significant.

cedar leaf, containing 60% thujone, was given intraperitoneally as a 10% solution in 95% ethyl alcohol. The rats were observed for 1 hour. Convulsions were recorded both as to incidence and severity: brief but definite bursts of clonic twitching were included in the total convulsions; while generalized, prolonged, tonic-clonic seizures were maximal. Other groups of 20 rats each were gastrically premedicated with single doses of isopropyl alcohol, 1250 mg/kg, diphenylhydantoin, 100 mg/kg, or phenobarbital, 50 mg/kg; and 1 hour later these rats were given the convulsants in the same doses as in the controls. The doses of isopropyl alcohol and phenobarbital were those found previously to be the maximal which would not produce undesirable motor depression.

In doses which did not cause motor ataxia, isopropyl alcohol compared favorably with phenobarbital in preventing and ameliorating the convulsions of metrazol. This alcohol was superior to phenobarbital in antagonizing thujone seizures; but the alcohol was less effective than phenobarbital against picrotoxin. Diphenylhydantoin afforded protection against picrotoxin convulsions only.

Summary. Analyses of blood isopropyl alcohol and blood acetone in relation to elevation of cortical threshold to electric stimulation suggest that the alcohol itself is responsible for the anticonvulsant effect. The ketonemia, nonetheless, may contribute a share. Other secondary and tertiary alcohols (2-butanol, 3-pentanol, and 2-methyl-2-butanol) effectively raised the cortical threshold, with or without acetonemia. Isopropyl alcohol was capable of antagonizing metrazol and thujone convulsions but was not effective against picrotoxin seizures.

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Therapy of Paroxysmal Pulmonary Edema by Anti-Foaming Agents (17858)

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In spite of accepted therapeutic measures, paroxysmal pulmonary edema still has a high mortality. For this reason, any new drug or aid may be of importance. It has long been known(1) that large amounts of fluid may be tolerated in the respiratory passages as long as no foam is formed. When the latter occurs, increased volume of the fluid and modified physical properties cause severe effects by blocking the small bronchi and adding the effects of anoxia to those already causing pulmonary edema. For this reason, a systematic study of several anti-foaming agents introduced into the respiratory passages was undertaken.

Material and method. The study was made in a series of 100 white, male rabbits weighing between 1.6 and 2 kg. Paroxysmal pulmonary edema was induced by the standard method previously used by the author(2) and slightly modified by Glass(3). Adrenalin Parke and Davis was injected slowly into the marginal vein of the ear, 2 cc of the one to one thousand solution being injected in one minute. The following anti-foaming substances were tested: (a) ether; (b) octyl alcohol;† (c) capryl alcohol;‡ (d) 95% ethyl alcohol; (e) sorbitan triolate (Span 85).§ The rabbits were placed into a box with a glass cover and each of the above drugs was sprayed into it at frequent intervals by means of an atomizer. The action of the drug was prolonged for 10 minutes prior to the injection of adrenalin and then until

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3. Glass, A., *Arch. Exp. Path. u Pharm.*, 1928, v136, 72.

† n-octyl alcohol, supplied by Eastman Kodak Co.

‡ methyl-n-hexacarbinol, supplied by Eastman Kodak Co.

§ This substance was obtained through the courtesy of the Atlas Powder Co.

TABLE I.
Influence of Various Anti-foaming Agents on Adrenalin Pulmonary Edema.

Drug	% of survival at LD_{50} (6 min.)	Avg survival (min.) and standard error*	Avg lungs/body ratio and standard error*	No. of animals	Beneficial effect
(Normal animals)	—	—	0.455 ± 0.021	4	—
Adrenalin (controls)	50	15.5	1.55 ± 0.035	12	—
Span 85	25	6	1.48 ± 0.094	4	0
Heavy alcohols (inhal.)	70	20.6	1.30 ± 0.098	10	x
Ether (inhal.)	100	29	1.15 ± 0.266	5	x
Morphine (subcut.)	90	35	1.01 ± 0.109	10	xx
Ethyl alcohol (inhal.) (subanesth. dose)	90	35	0.95 ± 0.092	20	xx
Ethyl alcohol (intrav.) (subanesth. dose)	60	18.6	1.29 ± 0.263	5	x
Ethyl alcohol (G-I) (anesth. dose)	100	33.5	0.76 ± 0.130	7	xxx
Morphine (subcut.) + ethyl alcohol (inhal.)	100	42.4	0.68 ± 0.048	10	xxxx
Morphine (subcut.) + oxygen under pressure	100	43.6	0.60 ± 0.051	5	xxxx

$$* \text{Standard error} = \sqrt{\frac{\Sigma\Delta^2}{(n)\ (n-1)}}$$

death of the animal. However, if the rabbit survived, at the end of 60 minutes it was sacrificed for control of the lungs. For this reason, the longest survival time was actually 60 minutes. In order to control the action of ethyl alcohol, this was also given intravenously in a subanesthetic dose (5 cc per kg of a 25% solution); by stomach or rectum in anesthetic dose (8-10 cc per kg of a 50% solution); or associated with morphine sulfate. The latter was given in dose of 10 mg per kg by subcutaneous injection; adrenalin was administered 30 to 40 minutes later. Ether was given in subanesthetic dose. Further controls were made with morphine and oxygen. Pure oxygen was given under a pressure of 3 to 4 cm H_2O through a tracheal cannula in morphinized rabbits. Adrenalin was injected soon after starting the oxygen jet.

Evaluation of the outcome was done in the following way: observation of the animal; possible outpour of foam from the mouth, nostrils or cut trachea; macroscopic appearance of the lungs; lungs/body ratio. The latter was obtained by dividing the weight of the lungs in grams by that of the animal in grams and multiplying the result by 100. Controls on normal rabbits showed that, by this method, an average ratio of 0.45 is obtained.

Results. *Span 85* did not change the severity of the edema and seemed to abbreviate the survival time. *Heavy alcohols* slightly increased the survival time and decreased the severity of the edema while subanesthetic doses of ether also helped somewhat. *Ethyl alcohol* by inhalation had a marked action, revealed by the fact that the average survival time was doubled and the average lungs/body ratio was decreased, from 3 times, to twice the normal. This effect of ethyl alcohol was as favorable as that of *morphine* (see table). Equivalent subanesthetic doses of ethyl alcohol by intravenous injection had a less favorable effect. On the contrary, larger doses of alcohol causing anesthesia, given by gavage or enema, had a marked favorable action, slightly superior to that of inhalation alcohol.

Combined therapy by means of morphine plus inhalation of ethyl alcohol or by morphine plus oxygen under pressure gave excellent results, revealed by the lack of edema and by a long survival time.

Discussion. The stability of a foam is based upon the character of the air-fluid interface, namely on the surface tension of the fluid. Any substance capable of modify-ing the surface tension in such a way as to decrease the foam, is called *an anti-foaming agent.* The favorable action of anti-foaming agents was revealed by the above mentioned experiments. However, this action was either enhanced or reduced by other properties of the drugs. Both Span 85 and heavy alcohols seemed to exert some unfavorable effect which neutralized their anti-foaming action. It is possible that coating of the mucosa or plugging of bronchioles by droplets of these fluids accounts for the result. Ether's anti-foaming effect was probably partly counteracted by its irritant action which increased bronchial and alveolar congestion. Ethyl alcohol proved to be the best. Controls with equivalent subanesthetic doses injected by vein and with larger, anesthetic doses given via g-i tract revealed that a twofold effect is obtained through the use of inhaled alcohol: one favorable action is local, and consists of the reduction of foam; the other is general and is based on its depressant effect on the nerve centers. This is revealed by the fact that, in order to obtain comparable effects, parenteral alcohol has to be given in much larger doses than when inhaled. The useful action of alcohol is revealed by the fact that it is equivalent to that of morphine: 9 out of 20 animals had slight or no edema of the lungs; 10 out of 20 survived more than 50 minutes; 19 out of 20 had no bloody foam from the nostrils.

Association of parenteral morphine with alcohol by inhalation gave excellent results. Statistical analysis was made of the data obtained in two series of experiments comparing alcohol by inhalation with alcohol by inhalation plus parenteral morphine. It was proven that statistically $t_{28} = 4.0$, which corresponds to a probability of less than 0.1%; therefore, the means of the two lungs/body ratios are different, and the combination of the two drugs is superior to each of them alone.

As shown by previous studies, air and oxygen under pressure are effective in the treatment of acute pulmonary edema caused by adrenalin(4,5). In our experiments, morphine plus oxygen under pressure saved a great number of animals. Comparison between this series and that treated by morphine plus alcohol by inhalation showed that the results obtained by using either of the two methods are equivalent. The combination of all 3 remedies, morphine, oxygen under pressure and alcohol vapors seems, therefore, to be indicated in clinical cases. The only reason why it was not tried in our experimental series is that, in adrenalin pulmonary edema, the results were already so good with 2 of the remedies that further improvement could not be expected through the addition of a third.

Summary. Experiments were performed with anti-foaming agents by inhalation in rabbits in order to decrease the severity of pulmonary edema caused by a standard dose of intravenous adrenalin. Poorly volatile drugs (heavy alcohols, Span 85) failed to exert any favorable effect. Ether gave only a slight improvement. Ethyl alcohol exerted an important favorable action. This action is partly due to the anti-foaming property of alcohol and partly to its action on the central nervous system. Combination of parenteral morphine with alcohol by inhalation gave the best results.

The author wishes to acknowledge the technical assistance of Miss Edith B. Shafer and is indebted to Dr. A. R. Goldfarb for statistical analysis.

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Determination of Gastric Acidity without Intubation by Use of Cation Exchange Indicator Compounds.* (17859)

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(Introduced by William S. McCann)

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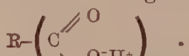
The present study is concerned with the use of cation exchange resins to produce cation exchange resin indicator compounds, which will permit the determination of gastric acidity without subjecting the individual to intubation.

The specific compound employed was prepared from Amberlite IRC-50 or Amberlite XE-96[†]. This type of synthetic cation exchange resin has the general formula of



$$\mathrm{R{-}}\left(\mathrm{C}\genfrac{}{}{0pt}{}{/\!/\,\mathrm{O}}{\backslash\,\mathrm{O^{-}H^{+}}}\right)_n .$$

When prepared in the sodium salt form, its sodium cations can be displaced by the hydrogen cations of a dilute hydrochloric acid solution or by those present in gastric juice. If a special cation is first combined with the cation exchange resin and this cation is displaceable only or mainly by hydrogen cations, is readily absorbed from the stomach or small intestine, is non-toxic and easily detectable in the blood, the urine, and/or the saliva, a test will be available for determining the presence of hydrochloric acid in gastric juice without subjecting the individual to intubation. If the special cation is displaceable to any extent by the cations of the secretions of the small intestine, the relationship between the time of oral administration of the indicator compound and the appearance of the indicator cation in the blood or urine, etc. must be taken into account. The indicator cation might be a dye, a radioactive material, or any other easily detectable substance.

Methods and results. The indicator compound called *quininium resin indicator compound* (IEC-QH)[‡] was prepared as follows: A batch of the cation exchange resin used was conditioned in the acid form. It was then allowed to react with an aqueous solution of quinine hydrochloride so that one g of the resin took up the quinine cations present in 20 mg of quinine hydrochloride.

A. *In vitro* studies. The following technic was used to test the efficacy of the hydrogen cations in dilute hydrochloric acid solutions and in gastric juice, as well as the cations in 0.7% solutions of sodium, potassium, calcium, and magnesium chloride in displacing the quininium cation from the quininium resin indicator compound: To 0.1 g of the IEC-QH in a test tube were added 10 ml of the solution to be tested. The temperature was kept constant at 37.5°C and the mixture was stirred with a slow stream of air. The presence of the quininium cation resulting from the displacement of this cation from the indicator compound was demonstrated approximately by the intensity of the fluorescence observed when the solution was exposed to ultraviolet rays. The quininium cation was displaced from the cation exchange resin indicator compound only when the pH of the dilute hydrochloric acid solution was 2.9 or less. It was displaced by the hydrogen ions of gastric juice below pH 3.01-3.20. Sodium, potassium, and calcium cations displaced the quininium cation only faintly while magnesium cations had more avidity in desorbing the quininium cation.

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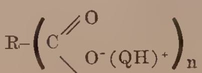
[*] This investigation is supported by special medical research funds.

[†] The authors are grateful to the Rohm & Haas Co. of Philadelphia, Pa., for the generous supply of these Amberlites and other types of resin used in this study.

[‡] IEC-QH will be used to designate the quininium cation resin indicator compound:



$$\mathrm{R{-}}\left(\mathrm{C}\genfrac{}{}{0pt}{}{/\!/\,\mathrm{O}}{\backslash\,\mathrm{O^{-}(QH)^{+}}}\right)_n$$

B. *In vivo* experiments. *In vivo* experiments were performed by administering the IEC-QH orally in 2 g doses with 50 cc of 7% alcohol, as the gastric stimulant, in patients in whom the gastric secretory response to alcohol had been determined previously by the usual intubation technic on a day other than that of the resin indicator test. About one-third of the liberated quininium cations will be excreted in the urine (1). Urine was collected before and one, 2, and 3 hours after the oral administration of the quininium indicator compound. From each sample the quinine was extracted by the ether-sulphuric acid technic described by Kelsey and Geiling(2). The cation indicator compound was administered orally to 63 individuals. In 34 of 38 patients with free gastric hydrochloric acid, quinine, as determined by the fluorescent (qualitative) technic was found in the urine excreted in the first hour after the administration of this compound, as well as in the second and third hours. With this same fluorescent technic none of the 25 achlorhydric patients showed the quinine in the first hour of urine excretion and 20 also failed to excrete it in the second hour.

Table I demonstrates the value of the quantitative photofluorometric technic in those patients in whom the qualitative fluorescent technic revealed quinine in the second and third hour and not in the first hour of urine excretion. Patients with no free gastric hydrochloric acid showed a very low photofluorometric reading in the second hour urine extract. Individuals with free gastric hydrochloric acid in whom the quinine did not appear in the first hour, but only in the second and third hours, revealed a high photofluorometric reading.

The data obtained in the studies *in vivo* show that it is now possible to divide proper subjects into 3 groups of gastric secretory response by examining the urine after the

TABLE I.

Comparison Between Fluorescent and Photofluorometric Methods of Determining Amount of Quinine in Urine of Individuals With and Without Free Gastric Hydrochloric Acid.

Case	Diagnosis	Gastric acidity in M.Eq./L 30 min. after alcohol (Intubation technic)		Intensity of fluorescence Hr of urine collection				Photofluorometric readings* Hr of urine collection			
		Free acid	Total acid	0	1st	2nd	3rd	0	1st	2nd	3rd
S.	Duod. Ulcer	93	101	—	+	++	++	3.0	15.0	42.3	69.4
P.	” ”										
W.G.	Diabetes mellitus	90	102	—	—	+	++	3.5	3.5	24.6	41.7
H.G.	Cholecystitis	0	8	—	—	—	†	3.6	3.7	3.9	†
O.	“Nervous indigestion”	0	12	—	—	tr	+	3.1	3.6	6.0	14.9
	“Achlorhydric diarrhea”	0	8	—	—	tr	tr	2.6	3.0	4.1	5.5

* These readings can be converted by appropriate tables to concentration values.
† No urine obtained.

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1. Goodman, L., and Gilman, A., The Pharmacological Basis of Therapeutics, The Macmillan Co., 1941, 910.

2. Kelsey, F. E., and Geiling, E. M. K., *J. Pharm. and Exp. Therap.*, 1942, v75, 183.

administration of IEC-QH with the fluorescent qualitative technic described.

(1) The gastric secretion of the group in whom the quininium cations appear in the urine during the first 2 hours after the administration of the indicator compound will have free hydrochloric acid. (2) The gastric secretion of the group in whom the quininium cation does not appear in the urine until the third hour after the administration of the indicator compound will have no free hydrochloric acid. (3) The group in whom the quininium cation is not excreted in the first hour but appears in the second hour will require quantitative photofluorometric examination of the urine extracts to determine the presence or absence of free gastric hydrochloric acid.

The use of this cation exchange indicator compound should find an important place in both clinical diagnosis and in preventive medicine.

Summary. The preparation of a cation exchange indicator compound called quininium resin indicator compound has been described. The rationale and method of its use have been stated.

In vitro tests have demonstrated that the quininium cation present in this compound will be displaced by the hydrogen ions of dilute hydrochloric acid solutions and of the gastric juice.

In vivo experiments have shown that, by administering this cation exchange resin indicator compound orally and noting the time of appearance of the quininium cation in the urine, the presence or absence of free hydrochloric acid in the gastric juice can be determined without subjecting the individual to intubation.

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Transmission and Multiplication of Newcastle Disease Virus (NDV) in Brains of Suckling Mice. (17860)

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Adult mice have generally been found resistant to Newcastle disease. Brandly *et al.* (1) concluded that NDV failed to multiply on intracerebral inoculation of weanling mice. Recently Reagan *et al.*(2) report transmission of Newcastle disease to adult mice, using virus from the 203rd hamster passage. The results of present investigations indicate that 3-day-old mice are almost all susceptible to at least 2 strains of NDV and that this infection is readily transmissible to successively older mice.

Materials and methods. The California strain of NDV (No. 11,914) was obtained from Dr. Erwin Jungherr of Storrs, Conn. Dr. Alfred S. Evans kindly supplied an Australian strain of NDV. Suspensions of these viruses were prepared in amniotic and allantoic (AA) fluids as described elsewhere (3). The Newcastle disease immune hen serum used as a standard was kindly supplied by Dr. Clarence H. Thompson, Jr. of the Bureau of Animal Industry, U. S. Department of Agriculture. Hen erythrocytes were employed in antihemagglutination tests as elsewhere described(3).

Mouse passages. The Australian and California strains were passed in essentially the same manner in separate series of Swiss albino mice. Virus suspensions were diluted in buffered saline (pH 7.6) and inoculated intracerebrally in amounts of 0.03 ml. Infected brain tissue was ground without

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1. Brandly, C. A., Moses, H. E., Jungherr, E. L., and Jones, E. E., *Am. J. Vet. Res.*, 1946, v7, 289.

2. Reagan, R. L., Lillie, M. G., and Brueckner, A. L., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 5.

3. Kilham, L., Jungherr, E., and Luginbuhl, R. E., *J. Immunol.*, 1949, v63, 37.

TABLE I.

Hemagglutination Titrations and Antihemagglutination Tests in Which AA Fluids from Embryonated Eggs, Inoculated with Mouse Brain Suspensions Were Used.

Strain of NDV	Mouse brain passage used for egg inoculation	AA fluids obtained from eggs inoculated with infected mouse brain: Hemagglutinin titers	Antihemagglutinin titers of standard serum
California	4th	1: 128	1: 8192
”	8th	1:1024	1:32768
Australian	8th	1: 512	1: 2048
”	10th	1: 128	1: 1024

abrasive for dilution and transfer. On the initial passage, 3-day-old mice received infected AA fluids diluted 10^{-1}. All mice inoculated became ill in 4 to 6 days. Weaned mice, 4 weeks old, failed to become ill when similarly inoculated. In subsequent passages, infected brains of the suckling mice were transmitted in dilutions of 10^{-2} or 10^{-3}, inoculated mice becoming ill in 2 to 3 days. The age of mice at time of inoculation was successively increased. Twelve- to 14-day-old mice were used for the 10th brain passages. Manifestations of Newcastle disease in afflicted mice were similar for both strains of NDV. Younger mice had humped backs and used their hind legs stiffly. As the disease progressed, especially in older mice, neurological manifestations were striking. Manifestations varied, some mice turning repeatedly in one direction while others had paralyses or uncontrolled movements of various limbs. Some mice had convulsions. Death occurred 2 to 3 days after the appearance of illness. Suspensions of brain tissue from each passage, as well as all egg fluids, were cultured for bacterial contaminants in thioglycollate and in meat infusion broths. Contaminants appeared on 2 occasions, but were eliminated by the addition of penicillin and streptomycin.

Passage of infected mouse brain suspensions in embryonated eggs. Two per cent suspensions of infected mouse brains along with penicillin and streptomycin were inoculated into allantoic sacs of 10-11-day-old eggs. The 4th passage brain suspensions of the Cal. NDV and 8th passage suspensions of the Aust. NDV killed all of the embryonated eggs inoculated with each within 44 hours. As presented in Table I the AA fluids when harvested had hemagglutinin titers of 1:128 for the Cal. NDV and of 1:512 for the Aust. NDV. Eighth mouse brain passage of the Cal. and 10th passage of the Aust. NDV were inoculated into a later set of eggs. The AA fluids of these eggs had HA titers of 1:1024 for the Cal. NDV and of 1:128 for the Australian. All of the infected AA fluids, when diluted in amounts containing 8 hemagglutinating units, gave substantial titers with the standard NDV-immune serum in antihemagglutination tests (Table I). These tests served to indicate that the agents passed were actually Newcastle disease viruses.

Infection of chicks with mouse brain suspension. Mouse brain suspensions from the 8th passage of Cal. NDV and 10th passage of Aust. NDV were inoculated intracerebrally into 2-week-old chicks in amounts of 0.05 ml. All chicks became ill and moribund within 2 to 4 days. They appeared hunched, with drooping wings, and had difficulty standing. Brains of moribund chicks were harvested and inoculated into 10-day-old embryonated eggs. Antihemagglutination tests against the standard serum indicated that the AA fluids of these eggs contained NDV.

Discussion. Australian and California strains of NDV have been transmitted through 10 passages in the brains of suckling mice. As both agents increased in virulence with passage, there apparently was an active multiplication of virus. The agents transmitted showed many features characteristic of NDV. They multiplied rapidly in embryonated eggs, killing them in less than 2 days and producing hemagglutinins in the AA fluids. These fluids yielded high titers when run in antihemagglutination tests with a standard NDV-immune serum from the Bureau of Animal Industry. Inoculated mice developed encephalitis suggestive of Newcastle disease in fowls. Viruses from infected mouse brains, produced Newcastle disease when inoculated intracerebrally into 2-week-old chicks. The agents transmitted were resistant to freezing and thawing, penicillin, and streptomycin. The ease with which 2 different strains of NDV are transmissible in the brains of suckling mice may have value in investigations of the immunology and other aspects of Newcastle disease. Further passages, now in progress, suggest that weanling and older mice are susceptible to NDV following preliminary passages already described.

Summary. Suckling mice of the Swiss albino strain are susceptible to Newcastle disease of fowls following intracerebral inoculation. This has been demonstrated by 10 consecutive passages of the California and of an Australian strain of NDV.

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Unique Physico-Chemical Properties of Japanese B Encephalitis Virus Hemagglutinin.* (17861)

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The discovery of the phenomenon of hemagglutination with the influenza viruses(1,2) has provided an important new tool for the study of certain basic properties of a number of viruses(3,4). No such hemagglutinins, however, could be demonstrated for the neuronotropic viruses affecting the human nervous system. Recently, the phenomenon has been found to occur with the virus variously known as the "Columbia S.K.," "M.M.," encephalomyocarditis (EMC), or Mengo encephalomyelitis(5-7) and with the GDVII strain of mouse encephalomyelitis(8). During a visit to the University of Leiden (Holland) in September, 1949, Prof. J. D. Verlinde informed one of us (A.B.S.) that he obtained the same results with the Nakayama strain of Japanese B encephalitis as with the "S.K.-M.M." virus, and that by hemagglutination inhibition tests it was indeed not possible to distinguish these viruses. Olitsky and Yager(6), however, shortly thereafter in reporting their own studies with the "S.K.-M.M." virus indicated that they obtained only negative results with the Japanese B encephalitis virus and a number of other viruses affecting the human nervous system. In an attempt to clarify this situation it was discovered that the virus of Japanese B encephalitis was indeed associated with a specific hemagglutinin. Its properties, however, were found to be so different from all others previously described that it became evident that Verlinde and DeBaan(7) were dealing not with the Japanese B encephalitis virus, originally obtained from this laboratory, but with

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[*] Aided by the Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board.

[†] Captain, M.C., U.S.A., on active duty.

1. Hirst, G. K., *Science*, 1941, v94, 22.

2. McClelland, L., and Hare, R., *Canad. Pub. Health J.*, 1941, v32, 530.

3. Hirst, G. K., *J. Exp. Med.*, 1950, v91, 161.

4. Burnet, F. M., *Lancet*, 1948, v1, 7.

5. Hallauer, C., 4th Internat. Cong. Microbiology, July 1947, Copenhagen, 1949, p. 257.

6. Olitsky, P. K., and Yager, R. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 719.

7. Verlinde, J. D., and DeBaan, P., *Ann. Inst. Pasteur*, 1949, v77, 632.

8. Lahelle, O., and Horsfall, F. L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 713.

the "S.K.-M.M." virus with which an unfortunate accidental contamination must have occurred.‡

The original positive results in this laboratory were obtained with centrifuged, unbuffered saline extracts of infected mouse brains and 0.25% suspensions of either sheep or chick cells, incubated at room temperature (over 22°C) or 37°C. Agglutination of the erythrocytes was evident only in the sedimentation pattern, the affected cells settling out in a shield over the entire round bottom of the tube, while the unaffected cells sedimented in a compact button as described by Salk(9) for the influenza viruses. Further experience with this system indicated, however, that the erythrocytes obtained from the same chicks at various periods after hatching or from different sheep not infrequently yielded negative or very poor results. Regular and reproducible results were obtained only with erythrocytes from chicks bled during the first 24 hours after hatching. No hemagglutination was obtained with human type "O" or *Macacus rhesus* erythrocytes. Extracts similarly prepared from uninfected mouse brains or from brains infected with the viruses of St. Louis (SLE), Western equine (WEE), Eastern equine (EEE), or Russian spring-summer (RSSE) encephalitis, or with the EMC (encephalomyocarditis) virus, yielded negative results with chick cells, which in simultaneous tests gave positive hemagglutination with the standard Nakayama and the more recently recovered Okinawa(10) and Korea(11) strains of Japanese B encephalitis virus.

When the phosphate-buffered saline (pH 7.6) used by Olitsky and Yager(6) was substituted for the unbuffered saline, we also obtained only negative results with the Japanese B virus. Preliminary tests indicated that in certain concentrations phosphate, citrate, borate and arsenate ions prevented hemagglutination, while oxalate, fluoride, tartrate and sulfate ions did not. It was found, however, that pH, rather than the ions present, was the determining factor in hemagglutination with this virus. The reaction occurred only in the narrow zone between pH 6.2 and 7.1, the optimum being between pH 6.5 and 6.8. Saline buffered with 0.01 M collidine prepared according to Gomori(12) or with 0.01 M borate yielded results very similar to those obtained with 0.01 M phosphate. The pH zone was narrower within this range and the activity very much lower when all the dilutions were first prepared in unbuffered 0.9% NaCl and the buffers of different pH added afterwards than when the dilutions were made directly in the buffered solutions of 0.9% NaCl. Hemagglutinin titers in unbuffered saline varied with the age of the preparation and various other factors, to be described elsewhere, but the marked enhancement of titer (from 1:60 to 1:5,120 shown in Fig. 1) occurred only when the dilutions of appropriately prepared extracts were made in the buffer and not when they were first made in saline and adjusted to the optimum pH and buffer concentration afterwards. It is also noteworthy that this enhancing action of appropriate pH and buffer concentration is obtained regularly only with erythrocytes of newly hatched chicks, and irregularly, not as well or not at all with those of chickens or sheep. Suitable experiments have shown that the hemagglutinin is not rapidly destroyed at a pH directly to the acid or the alkaline side of the required zone but rather that it does not combine with the erythrocyte receptors except in the narrow pH zone indicated above. Hemagglutination cannot be reversed merely by resuspension in solutions of a pH outside the required zone.

Continued experience with extracts prepared from mouse brains infected with the virus of Japanese B encephalitis yielded highly variable and occasionally negative results which suggested that the infected mouse brain

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‡ In a recent personal communication Prof. Verlinde indicated that he agrees with this conclusion.

9. Salk, J. E., *J. Immunol.*, 1944, v49, 87.

10. Sabin, A. B., *J. Am. Med. Assn.*, 1947, v133, 281.

11. Sabin, A. B., Schlesinger, R. W., Ginder, D. R., and Matumoto, M., *Am. J. Hyg.*, 1947, v46, 356.

12. Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1946, v62, 33.

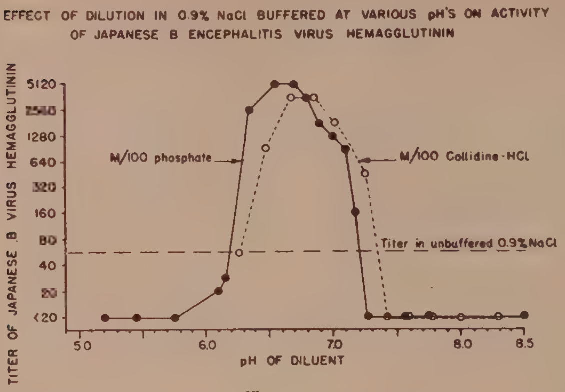

FIG. 1.

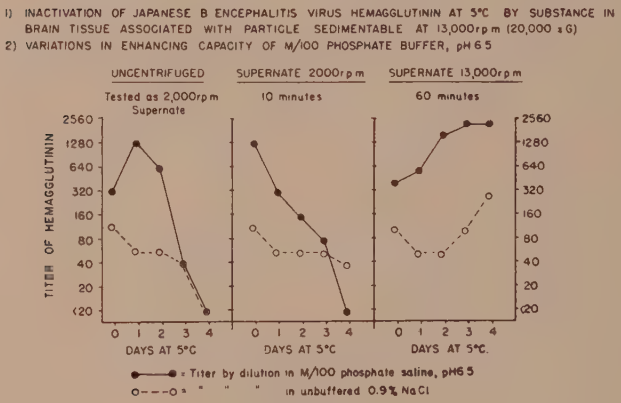

FIG. 2.

suspension was a highly complex, dynamic mixture in which a variety of reactions influencing the ultimate activity of the hemagglutinin were in progress. A systematic investigation of hemagglutinin activity in various fractions of a 20% suspension of infected mouse brains in unbuffered saline at various time intervals after preparation yielded information which permitted the separation of the hemagglutinin in a form which remained stable at 4 to 6°C for many weeks. Two findings require special mention: (1) that a substance associated with particles sedimentable at approximately 20,000xG (13,000 r.p.m. for 1 hour on the Sorvall SS-1 centrifuge in the cold room) inactivates the hemagglutinin at about 5°C in 3 to 4 days; (2) that the 13,000 r.p.m. supernate increases in activity after storage at about 5°C for several days and then remains stable for 8 weeks or longer (Fig. 2). Based on these studies, the following optimum method for preparing Japanese B encephalitis virus hemagglutinin was devised.

Mice inoculated intracerebrally with 10% virus suspension are bled and sacrificed when they are almost prostrate (dead mice are not used) and a 20% suspension of the brains, washed free of blood, in unbuffered, 0.9% NaCl is prepared by grinding with alundum in a mortar. The Waring blendor is not used because severe agitation is deleterious to the hemagglutinin. The brains are not suspended in buffered saline of optimum pH because this results in extraction of larger amounts of inhibitor or inactivating substances. After storage of the uncentrifuged brain suspension at 4 to 6°C for not less than 3 hours and not more than 12 hours, it is centrifuged either at 2,000 r.p.m. for 10 minutes or preferably at 13,000 r.p.m. in the cold for 10 minutes. The supernatant liquid is then centrifuged at 13,000 r.p.m. for 1 hour in the cold room. The 13,000 r.p.m. supernate is then stored in an ordinary refrigerator at 4 to 6°C. Although high activity is often demonstrable immediately, the maximum titer of hemagglutinin may not appear for 3 to 10 days. The Nakayama, Okinawa and Korea strains of Japanese B encephalitis have yielded similar results with this method of preparation. Extracts of brains from uninoculated mice or from mice inoculated with the EMC, WEE, EEE, SLE or RSSE viruses prepared and stored in the same manner have given completely negative results with chick cells.

The marked instability of *various dilutions* of this hemagglutinin at temperatures above 4 to 6°C is especially noteworthy. The results shown in Fig. 3 are typical for the inactivation observed when a series of dilutions prepared in 0.01 M phosphate saline, pH 6.5, are stored at different temperatures for 30 or 60 minutes before addition of the 0.25% chick erythrocyte suspension. Glycine in 0.25% to 1% concentration in phosphate-buffered saline, pH 6.5, without significantly affecting the pH depressed the titer and did not alter the stability of the diluted hemagglutinin at room temperature. Rapid freezing at about –80°C (solid CO_2 and alcohol) and thawing, as well as lyophilization, have a different effect on the 20% uncentrifuged brain suspension and the 13,000 r.p.m. supernate. The uncentrifuged suspension retains its activity after a single freezing and thawing but loses it completely after being frozen and thawed 5 times in rapid succession, as well as after lyophilization. The 13,000 r.p.m. supernate, on the other hand, loses all of its activity after a single freezing and thawing, but retains approximately 50% of its activity after lyophilization. Mere bubbling of nitrogen gas through a 1:80 dilution of hemagglutinin in saline buffered at pH 6.8 with 0.01 M borate, kept in an ice bath, was enough to inactivate 75% of it in 5 minutes and 96% in 20 minutes, without significantly altering the pH.

LOSS OF ACTIVITY OF JAPANESE B ENCEPHALITIS VIRUS HEMAGGLUTININ DILUTED IN M/100 PHOSPHATE SALINE, pH 6.5, AT 24°C AND 37°C.

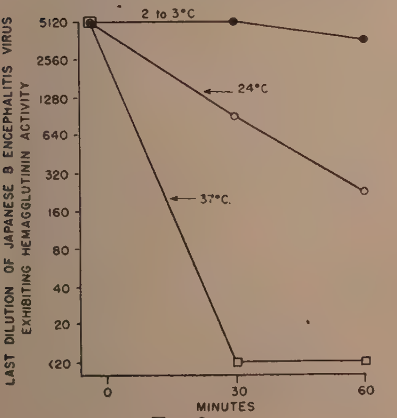

FIG. 3.

The fact that the 13,000 r.p.m. supernates exhibited high activity suggested that the particle size of much of the Japanese B encephalitis hemagglutinin was less than 100-150 mμ. Centrifugation of the 13,000 r.p.m. supernates on the Spinco preparative, vacuum ultracentrifuge[§] for 1 hour removed 75% of the hemagglutinin from the entire supernatant liquid at 21,740 r.p.m. (average 27,950xG), and practically 100% at 31,440 r.p.m. (average 58,300xG). The hemagglutinin would thus appear to be a macromolecular substance not smaller than the infective particle of Japanese B encephalitis virus, which by gradocol membrane filtration was previously found to be in the range of 15-22 mμ. However, several gradocol membrane filtration tests with hemagglutinin diluted in 0.01 M phosphate buffered saline, pH 6.5, or in unbuffered saline yielded data which were not in agreement with those obtained by centrifugation. The filtrates through membranes with an average pore diameter (A.P.D.) of 260 mμ or greater contained hemagglutinin while those from membranes with an A.P.D. of 200 mμ or less did not. One cannot be certain from the available data that the infectious particle and the hemagglutinin are identical, although they suggest that much of the hemagglutinin may exist in an aggregated or polymerized form which is larger than the infectious particle. This larger component may either represent the precursor or other modified form of the infectious particle, or a specific product of infection unrelated to it.

[§] We are indebted to Drs. J. E. Smadel and J. Warren of the Army Research and Graduate School for putting this centrifuge at our disposal, and to Dr. Warren and Capt. M. Weil, M.C., for their help with the tests.

Many experiments on the nature of the combination between the hemagglutinin and the chick erythrocytes suggest that hemagglutinin (H) combines with receptors (R) on the erythrocytes in an equilibrium type of reaction which may be expressed as:

$$H_x + R_y \rightleftharpoons H_xR_y$$

The data suggest that the number of R on a single erythrocyte combined with H varies with the concentration of H and the number of erythrocytes in the mixture, and that a minimum number of R on each cell must be combined with H to result in hemagglutination. Thus, in a solution properly buffered for optimum dispersion of H and combination with R, the hemagglutinin titer diminishes in a constant ratio as the concentration of erythrocytes increases (Fig. 4). When a series of tubes containing varying amounts of erythrocytes agglutinated by decreasing amounts of hemagglutinin (H) are repeatedly shaken at room temperature or at 37°C, the agglutination is reversed first in the mixtures with the smallest amount of H, while in the mixtures containing the largest concentration of H reversal occurs when the number of erythrocytes is large but not when it is small (at least not during a period of 48 hours). There is no evidence that the Japanese B encephalitis hemagglutinin is associated with a receptor destroying enzyme as is the case with influenza and certain other viruses. The reversal of agglutination here is due rather to the rapid destruction of unbound hemagglutinin (H) at room temperature and 37°C with the result that the equilibrium in the above reaction is more in the direction of dissociation of H_xR_y with the liberation of free "R" groups on the erythrocytes. The erythrocytes with the smallest number of R originally combined thus revert to their original state first. That the receptors (R) are unaffected in this reaction is evident from the fact that the erythrocytes are readily reagglutinated by the addition of more hemagglutinin (H). It may be worth noting here that in several simultaneous experiments, the enzyme or enzymes of *Vibrio cholerae*, as was expected, destroyed the receptor for the influenza virus on chick erythrocytes, but were without effect on the receptor for the hemagglutinin of Japanese B encephalitis virus.

INFLUENCE OF CONCENTRATION OF CHICK ERYTHROCYTES ON TITER OF HEMAGGLUTININ DILUTED IN DIFFERENT CONCENTRATIONS OF PHOSPHATE SALINE AT pH6.5 AND IN UNBUFFERED 0.9% NaCl

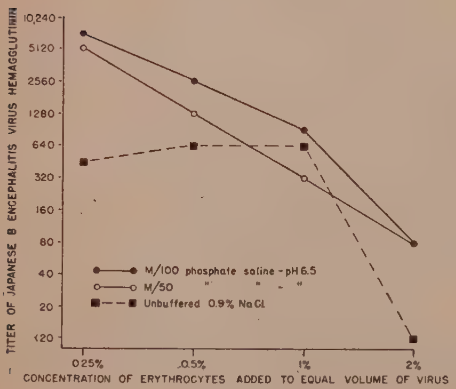

FIG. 4.

Normal human, rabbit, monkey and mouse sera were found to be capable of inhibiting hemagglutination by the Japanese B encephalitis virus. The inhibition in higher dilutions was due neither to the buffering capacity of the serum nor to any dialyzable component, and was only partly reduced by boiling for 15 minutes. The inhibitor does not act on the erythrocytes but appears rather to combine with the hemagglutinin. When the hemagglutinin is diluted in appropriately buffered saline, the inhibition titer is directly proportional to the concentration of hemagglutinin (Fig. 5); this proportionality cannot be demonstrated with hemagglutinin diluted in unbuffered saline. The available data furthermore suggest that the principal inhibitor in normal serum may represent cell receptor (R) in solution or a substance hav-

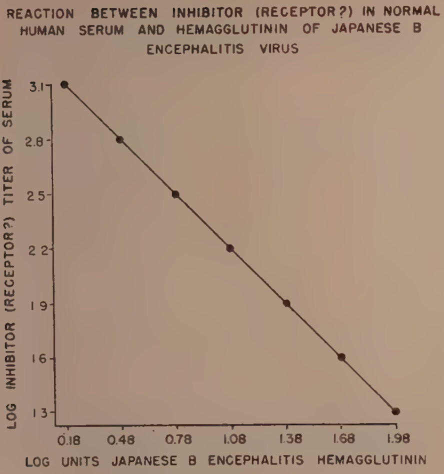

Fig. 5.

ing an affinity for the same combining groups on H. Thus, by the addition of diluted normal serum (free R?) it is possible to remove some of the H from the R on the erythrocytes and to reverse the agglutination of cells which had combined with a small amount of H (2 to 8 units). Although it was found that hyperimmune animal sera and sera from human beings recently recovered from Japanese B encephalitis generally possessed higher inhibitory titers than normal sera, the existence of a specific antibody for this hemagglutinin was established by experiment. After preliminary bleeding, one group of mice was given a series of inoculations of normal mouse brain suspension, another mouse brains infected with the virus of Western equine encephalitis (WEE), and a third with Japanese B encephalitis virus. The inhibitory titers of the preinoculation and postinoculation sera were identical in the groups which received normal mouse brain or the WEE virus, but increased 4 to 8-fold in the mice inoculated with the virus of Japanese B encephalitis. Neither normal sera nor hyperimmune Japanese B virus sera were capable of inhibiting the hemagglutination of sheep erythrocytes by the EMC (encephalomyocarditis, M.M., Columbia S.K., Mengo encephalomyelitis) virus, thus providing additional evidence that Verlinde and DeBaan(7) were not working with Japanese B encephalitis virus when they reported that these viruses could not be distinguished from one another by hemagglutination inhibition tests.

However, since the inhibitory titers of normal sera were high and variable, the hemagglutination inhibition test could not be used as a simple indicator of infection with the Japanese B encephalitis virus until some means could be found of removing the normal inhibitor without affecting the specific antibody. Periodate and several strong proteases had no effect on the normal inhibitor. The lecithinase in highly potent, purified *Clostridium perfringens* toxin, in the presence of calcium, changed only the heat stability of the normal inhibitor permitting its destruction by boiling. Extraction with lipid solvents (ether, benzene, chloroform, ethylene chloride) and precipitation and extraction with acetone or alcohol-ether mixtures were tried to test the hypothesis that the inhibitor (or receptor) might be a phospholipid or lipoprotein. It proved possible to remove the normal inhibitor to varying extent by these methods from rabbit serum and human serum but only partially from mouse and monkey serum. Simple shaking of 1 ml of fluid human or rabbit serum in 4 to 12 ml of chloroform yielded the best results with the removal of approximately 95% of the normal inhibitor, but leaving the specific antibody in seemingly undiminished titer. The results obtained with a number of normal human sera and the acute and convalescent sera of 3 unvaccinated Americans who contracted Japanese B encephalitis in Korea(11), shown in Table I, indicate that the simple chloroform extraction technique makes it possible to use the hemagglutination inhibition test for diagnostic purposes and as an indicator of previous infection with the virus of Japanese B encephalitis.

During the course of investigations on the possible role of iron-binding substances as inhibitors it was found that partially purified preparations derived from cultures of *Clostridium histolyticum* and *Clostridium lentoputrescens* (but not from *Clostridium perfringens*)‖ were capable of inhibiting hemagglutination by the Japanese B virus in final dilutions as high as 1:1,000,000 after boiling

TABLE I.
Separation of Normal Inhibitor from Specific Antibody in Human Sera by Extraction with Chloroform.

Source of serum tested		Inhibition titers* vs 6 units of Japanese B hemagglutinin	
		Untreated serum	Chloroform-treated
Pool of 2 normal American children		160	10§
Normal American adult—L.		320	10
” ” ” —W.		640	10
American patient 2†— 4 days after onset		640	320
15		320	320
” ” 3 4 ” ” ”		640	20
13 ” ” ”		640	320
” ” 4 4 ” ” ”		160	20
10 ” ” ”		640	320
25 ” ” ”		320	320
Japanese adults from Sapporo.	SA—2E	40‡	10§
No history of encephalitis	SA—7E	160	10
No neutralizing bodies for	SA—8E	20	10
Japanese B virus	SA—9E	320	<10
Japanese adults from Okayama.	OK—E5	40	40
No history encephalitis.	OK—E7	80	40
Neutralizing antibodies for Japanese	OK—E9	40	20
B virus present, suggesting inapparent infection in the past	OK—E10	40	20

* Highest original dilution of serum (0.25 ml) added to hemagglutinin (0.25 ml) and 0.25% suspension of chick erythrocytes (0.5 ml) which prevented hemagglutination.

† These are unvaccinated American adults who contracted Japanese B encephalitis in Korea in 1946. For ease of comparison, the same numbers are used as in the communication in which these cases are reported(11).

‡ The inhibition titers in 6 of the 8 Japanese sera tested are distinctly lower than any encountered in American sera tested thus far.

§ It has proved possible to reduce these normal inhibitory titers to less than 5 by adjusting the chloroform-treated serum to pH 6.6 with 0.01 N HCl.

for 15 minutes. The effect of these preparations was shown to be on the hemagglutinin and not on the receptors of the erythrocytes. Further purification of these substances by Dr. A. A. Tytell indicated that the inhibitor was associated with certain nucleic acid or protein components which were different from the proteinase. During the course of this work it was also found that in suitably buffered solution diethyl dithio carbamate (in a final concentration of about 1:2,000) was capable of inhibiting the hemagglutination, while the other, simple organic substances, 8-hydroxyquinoline and α, α'dipyridyl which also bind heavy metals, although not as well in the presence of proteins, were without effect in 20 times greater concentration.

In studying the effects of various metallic ions, it was discovered that $ZnSO_4$ in minute concentration (from 0.03 to 1.92 μg per unit depending on dilution) was capable of inhibiting hemagglutination by the Japanese B virus hemagglutinin, while $FeSO_4$ $MnSO_4$ and $MgSO_4$ were without effect. Bichloride of mercury and copper sulfate, beyond M/70,000, because in greater concentration they produced hemagglutination or hemolysis by themselves, were without effect on the hemagglutination produced by the virus. It was found that the inhibitory concentrations of $ZnSO_4$ (from M/4,000 to M/28,000 depending on the concentration of hemagglutinin) were without effect on the erythrocytes, but reacted rather with the hemagglutinin. The influenza virus hemagglutinin proved to be unaffected by

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‖ We are indebted to Dr. A. A. Tytell of the Department of Biochemistry for putting these preparations at our disposal.

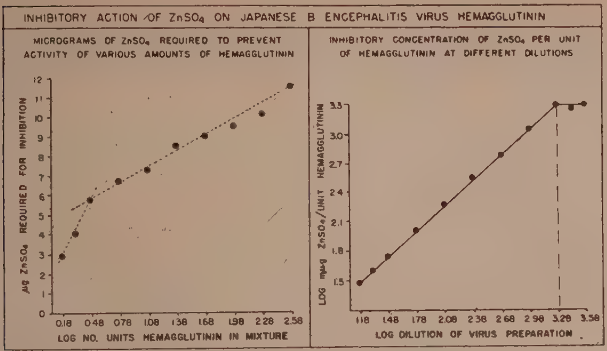

Fig. 6.

M/4,000 $ZnSO_4$. The reaction between $ZnSO_4$ and the Japanese B virus hemagglutinin (H) was found to be of the equilibrium type, which may be expressed as:

$$H_x + Zn_y \rightleftharpoons H_xZn_y$$

It proved possible to reverse this reaction by dilution and by the careful removal of Zn from the mixture with H_2S, which also indicated that the hemagglutinin was not denatured in the process. The amount of $ZnSO_4$ required to inhibit varying amounts of H was proportionately considerably greater when H was in highly dilute solution than when it was more concentrated (Fig. 6). It could also be shown that different amounts of $ZnSO_4$ were required to inhibit the same amount of H contained in different volumes, which may either be due to a disaggregation, "unfolding," depolymerization or dissociation of H upon dilution resulting in the appearance of more of the groups which combine with zinc, or to some other change in the kinetics of the reaction. All the experiments with zinc were carried out in 0.01 M collidine buffer (pH 6.5) or 0.01 M borate buffer (pH 6.8).

The unique properties of the Japanese B virus hemagglutinin discovered in this investigation indicate the lines along which the search for similar hemagglutinins may profitably be made in nervous tissue infected with the poliomyelitis and other encephalitis viruses. What is most important to remember is that the physico-chemical properties and biophysical state of the hemagglutinin may be totally different from that of the infectious particle, and that each virus may have its own narrow zone of pH for combination with receptor, and each be associated with its own special variety of inhibitors and inactivators. Utilizing chick, sheep, rhesus and human type "O" erythrocytes and diluents buffered at pH 5.5, 6.5, 7.5 and 8.5, a preliminary survey has been made of other neurotropic viruses and specific hemagglutinins have already been found for the West Nile and Russian Spring-Summer encephalitis viruses. The West Nile hemagglutinin became demonstrable with chick and sheep erythrocytes at pH 7.5 and the RSSE hemagglutinin only with sheep erythrocytes at pH 7.5, and both of these hemagglutinins gave negligible or negative reactions at pH 5.5, 6.5 and 8.5. The other properties of these hemagglutinins are now under investigation and appear to be different from those reported here for the hemagglutinin of the Japanese B virus.

Summary. A specific hemagglutinin was recovered from mouse brains infected with the virus of Japanese B encephalitis, and it was found to possess properties different from all other viral hemagglutinins described thus far. Stable preparations of high potency were obtained after removal of an inactivating substance by differential centrifugation. The hemagglutinin combined with the receptors on the erythrocytes in a very narrow pH zone, the optimum being between 6.5 and 6.8. Marked enhancement of activity was obtained on dilution in certain buffers of optimum pH. No receptor destroying enzyme was found either in the infected mouse brains or in

cultures of *Vibrio cholerae,* which destroyed the receptors for influenza virus. $ZnSO_4$ in minute amounts inhibited the hemagglutinin of the Japanese B virus but not that of influenza virus. The inactive zinc-hemagglutinin complex was reactivated by dilution or removal of zinc with H_2S. The inhibitor present in normal human serum was removed by shaking with chloroform, while the specific hemagglutination inhibiting antibody was unaffected, thus providing a simple test for diagnosis of infection with the Japanese B virus. Specific hemagglutinins, with properties different from that of the Japanese B virus, have also been found for the West Nile and Russian Spring-Summer encephalitis viruses.

Addendum. Since this paper was submitted for publication, work carried out in association with Drs. J. Warren and M. Weil of the Army Medical School, indicated that prolonged passage of the Nakayama strain of Japanese B encephalitis virus in mice resulted in complete loss of its hemagglutinating activity. Tests on 6 additional strains of Japanese B encephalitis virus, with relatively few passages in mice, revealed that all possessed a hemagglutinin similar to that described here for the Nakayama, Okinawa and Korea strains. Since the SLE, WEE, and EEE strains of virus we had used, had all had hundreds of mouse passages for many years, we repeated the work with freshly isolated strains of these viruses and have succeeded in demonstrating a specific hemagglutinin for chick and sheep cells at pH 6.5 to 7.0 with 3 strains of SLE, but thus far not with WEE or EEE. This work will be reported in detail in forthcoming publications.

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A Method for the Determination of Sodium Ferrocyanide at Low Concentrations in Body Fluids.* (17862)

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Gersh's(1) histochemical studies in the mammalian kidney on the distribution of ferrocyanide established its exclusive glomerular excretion. Renal clearances of ferrocyanide have been measured in the dog with plasma concentrations from 60-200 mg%. In one study the ferrocyanide was determined as Prussian blue(2); in a second as ferricyanide(3). These methods are accurate only at these high plasma concentrations. When human subjects were used, nephrotoxic reactions were encountered even with plasma concentrations of 3.3 to 43.6 mg%. The analyses were performed by hydrolysis and recovery of HCN(4). This time consuming method has lowered the working range of ferrocyanide concentrations, but in order to avoid the possible danger of toxic reactions, we have devised an accurate method sensitive enough to measure sodium ferrocyanide concentrations of 5-15 mg%. By the acid mixture described below, ferrocyanide is oxidized to ferricyanide and allowed to react with ferrous sulfate to form Turnbull's blue. The per cent transmittance is measured at 700 millimicra in the Coleman, Jr. spectrophotometer. The calibration curves show that the reaction follows Beer's law, and do not vary significantly from day to day. The color is

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* Aided by a grant from the National Heart Institute, United States Public Health Service.

1. Gersh, I., and Stieglitz, E. J., *Anat. Rec.*, 1934, v58, 349.

2. Van Slyke, D. D., Hiller, A., and Miller, B. F., *Am. J. Physiol.*, 1935, v113, 611.

3. Berliner, R. W., Kennedy, T. J., Jr., and Hilton, J. G., *Am. J. Physiol.*, 1950, v160, 325.

4. Miller, B. F., and Winkler, A., *J. Clin. Invest.*, 1936, v15, 489.

TABLE I.
Recovery of Various Concentrations of Sodium Ferrocyanide Added to Serum and Urine.

Amt $Na_4Fe(CN)_6$ added to serum and urine mg%	No. of single determinations	Mean recovery mg%	Stand. Dev. from mean, mg%
5	14	4.96	± 0.10
10	15	9.96	± 0.28
15	15	14.88	± 0.38
20	13	20.03	± 0.38

stable within 1% transmission for at least one hour after the addition of the ferrous sulfate.

Reagents. 1. *Sodium ferrocyanide.* Recrystallized in 95% ethyl alcohol. Standard solutions of 5, 10, 15, 20 and 25 mg% are prepared from this salt. 2. *Acid mixture.* 35 ml concentrated nitric acid and 40 ml 60% perchloric acid are added to 425 ml distilled water. 3. *Trichloroacetic acid.* 20%. 4. *Ferrous sulfate solution.* $FeSO_4 \cdot 7H_2O$, fine crystal, c.p. is added to saturation in 1N H_2SO_4. One part of this is added to 5 parts of the duponal solution and 7-10 drops of concentrated hydrochloric acid are added per 100 ml of this mixture. If the ferrous sulfate-duponal solution becomes turbid, it should be filtered; the solution remains clear after this filtration. 5. *Duponal solution.* Approximately 25 mg of duponal per liter of distilled water.

Procedure. Two ml of ferrocyanide standard, serum or urine, in proper dilution, are pipetted into a 20 x 150 mm ignition tube and 6 ml of distilled water added and thoroughly mixed with a footed stirring rod. Eight ml of acid mixture are then added with gentle stirring and the mixture allowed to stand 10-30 minutes with occasional stirring. After the addition of 2 ml of 20% trichloroacetic and further stirring, the tube is stoppered and centrifuged at 2500 r.p.m. for 15 minutes. Two ml of water treated in the same way provide a reagent blank. One ml of the ferrous sulfate-duponal solution is added to a 5 ml aliquot of the supernatant solution and the intensity of the blue color is then determined with a Coleman, Jr. spectrophotometer at a wave length of 700 millimicra. The reagent blank is set at 100% transmission.

Results. Recoveries of added sodium ferrocyanide to serum and urine in amounts of 5, 10, 15 and 20 mg%, give mean recoveries of 4.96, 9.96, 14.88 and 20.03 mg% respectively with standard deviations ranging from ±0.10 to ±0.38.

Conclusion. The above described procedure is a satisfactory method for measuring the sodium ferrocyanide concentration of body fluids at concentrations of 5 mg% or more.

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Sickle Cell Anemia, Blood Viscosity and Sodium Tetrathionate.[*] (17863)

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A decrease in blood viscosity, caused by the intravenous injection of sodium tetrathionate, has been reported by Theis and Freeland(1). This decrease apparently is accompanied by an increase in oxygen satura-

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[*] Aided by a grant from G. D. Searle and Co.

1. Theis, F. F., and Freeland, M. R., *Arch. Surg.*, 1940, v40, 190.

TABLE I.

	Viscosity Difference—Sec. (CO_2—O_2)			Hemoglobin—g %			Plasma protein—g %		
Patient	Before	1-4 hr after	24 hr after	Before	1-4 hr after	24 hr after	Before	1-4 hr after	24 hr after
				Whole Blood.					
W.P.	81.9	49.7							
T.G.	63.0	61.5							
M.	12.7	5.5	4.8	8.13	6.25	6.66	7.49	7.82	7.47
A.P.	13.0	8.7	10.6	7.77	7.10	8.09	7.26	6.20	6.74
I.G.	13.1	7.3		8.67	8.22		6.23	6.77	
S.	11.1	10.7	4.7	7.01	7.24	6.79	7.25	6.88	7.56
C.	15.3	17.0	11.8	9.56	8.98	9.96	7.46	7.74	6.86
M.G.	11.6	3.5		10.1	10.1		8.42	8.42	
J.	7.4	11.7		7.24	7.46		6.79	7.14	
A.	10.5	9.1	4.0	8.49	7.46	8.09	8.65	8.65	8.44
				Whole Blood Treated *in Vitro*.					
E.M.	3.8	4.1		6.21	6.21				
				Serum					
J.	0	0	0					8.31	7.75
				Serum Treated *in Vitro*.					
L.	0	0							

tion. The pathogenesis of sickle cell anemia, while yet obscure, seems to be associated, according to Pauling, *et al.*(2) with an abnormality in the structure of the hemoglobin molecule. This abnormality is reflected by changes in the shape of the erythrocyte, under reduced oxygen tension, which cause an increase in blood viscosity, thus setting up a vicious cycle, which may terminate in a state of crisis. The purpose of this investigation was to determine whether sodium tetrathionate would break the above mentioned vicious cycle by decreasing the viscosity of the blood of sickle cell anemia patients.

It has been shown(3) that by using an Ostwald type viscosimeter the relative viscosity of blood samples, after exposure to oxygen and carbon dioxide, can be expressed in seconds and that the difference, expressed in seconds, should be a fairly accurate measure of the viscousness of the blood of a patient with sickle cell anemia in crises. Simple measurements of absolute viscosity were deemed unsatisfactory in that changes in protein concentration or water balance, hemolysis or peptization of serum proteins would make the evaluation of results difficult. The use of the "difference value" simply uses the oxygenated blood as a control and thus eliminates, to a great extent, factors other than those pertaining to the erythrocyte itself.

Twelve patients, with definitely proved sickle cell anemia in crisis, or seemingly approaching crisis, were given sodium tetrathionate[†] by vein. Relative viscosity under carbon dioxide and oxygen, hemoglobin, and serum protein determinations were made, before and after the drug, upon a number of successive days. By reference to Table I, it is apparent that sodium tetrathionate does reduce the viscosity of blood of patients with sickle cell anemia. This reduction in viscosity is not due to hemolysis or destruction of cells, nor to a peptization of the serum proteins as can be seen from the data presented, in that no significant change occurred in hemoglobin or protein concentration.

No theory as to the mode of action of the tetrathionate can yet be formulated. It has no effect upon whole blood or serum *in vitro.* It has no action upon serum when injected intravenously. Seemingly its action is asso-

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2. Pauling, L., Itano, H. A., Senger, S. J., and Wells, I. C., *Science*, 1949, v110, 543.

3. McCord, W. M., Kelly, W. H., Switzer, P. K., and Culp, F. B., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 19.

[†] Obtained from G. D. Searle and Company as "Tetrathione."

ciated with the metabolic processes of the blood and may modify or affect the structure of the hemoglobin molecule. Pauling, *et al.* (2) used tetrathionate in a buffer system to prevent formation of methemoglobin during electrophoretic analytical procedures and indicates that tetrathionate did not affect the structure of "sickle cell hemoglobin", during these measurements. We propose to attempt to detect changes in the structure of the "sickle cell hemoglobin" in patients after treatment with tetrathionate. Clinical observation in regard to the use of this drug will be reported at a later date.

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Confirmation of Presence of a Gastric Secretory Depressant in Gastric Juice of Humans. (17864)

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In a series of papers Brunschwig and his co-workers(1-5) have reported finding a gastric secretory depressant in gastric juice of human beings. The gastric juice tested was secreted in response to subcutaneous injections of histamine after an overnight fast. Samples of the juice or alcoholic precipitates of the juice were injected into dogs with subtotal, wedge, or Heidenhain types of gastric pouches while these were secreting in response to frequently repeated feedings of small amounts of meat. Although inhibition of secretion sometimes occurred during tests of acid gastric juice, it was observed more frequently after the injection of achlorhydric gastric juice and especially when the achlorhydria was associated with pernicious anemia or cancer of the stomach. Using a quantitative method for the determination of inhibition of gastric secretory activity(6), we have confirmed the finding of Brunschwig and co-workers, that intravenous injections of alcoholic precipitates of achlorhydric gastric juice from human beings produce pronounced inhibition of gastric secretion in dogs.

Methods and procedure. Gastric juice was collected from human beings after an overnight fast by means of a Sawyer tube and intermittent suction. Histamine in a dose of 0.01 mg per kilo of body weight was used as the secretory stimulant. Achlorhydric gastric juice was collected from 2 groups of individuals: (1) 5 patients with pernicious anemia and (2) 8 persons who had no abnormality of the hemopoietic system and on whom roentgenographic studies of the stomach and duodenum were negative. Gastric juice containing acid was obtained from 9 individuals on whom tests of the stomach and upper part of the intestine revealed normal function. When the volume of juice obtained was small, samples from individuals in the same group were pooled. Alcoholic precipitates of the juice were prepared according to the method of Brunschwig and co-workers(3) by adding ethyl alcohol to the gastric juice until the concentration was 80%. After standing overnight the clear supernatant fluid was siphoned off, and the precipitate in the residue was collected by centrifugation. The

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1. Brunschwig, A., van Prohaska, J., Clarke, T. H., and Kandel, E. V., *J. Clin. Invest.*, 1939, v18, 415.

2. Brunschwig, A., Clarke, T. H., van Prohaska, J., and Schmitz, R. L., *Surg., Gynec., and Obst.*, 1940, v70, 25.

3. Brunschwig, A., Schmitz, R. L., and Rasmussen, R., *J. Nat. Canc. Inst.*, 1941, v1, 481.

4. Brunschwig, A., Clarke, T. H., van Prohaska, J., and Schmitz, R., *Ann. Surg.*, 1941, v113, 41.

5. Brunschwig, A., Rasmussen, R. A., Camp, E. J., and Moe, R., *Surgery*, 1942, v12, 887.

6. Code, C. F., Livermore, G. R., Jr., Ratke, H. V., and Blackburn, C. M., (Abstr.), *Am. J. Physiol.*, 1948, v155, 430.

TABLE I.
Gastric Secretory Inhibition Produced by Alcoholic Precipitates of Achlorhydric Gastric Juice from Patients with Pernicious Anemia.

Precipitate tested*			Assay		
Batch	Dose, mg	Dog	Control rate of secretion, % of max.	% inhibition	Max. rise of R.T.† °F
A	25	D	41	60	1.4
A	10	C	45	86	1.9
B	10	A	56	64	0.7
B	10	D	54	63	1.1

* For explanation see text.
† R.T. = Rectal temperature.

precipitate was dried in air, ground to a fine powder and weighed samples were prepared for testing by grinding in a mortar with physiologic saline solution or mixing in a Waring blendor for 5 minutes. The resulting suspension, in which some of the material was in solution, usually was administered intravenously to the test animals without further treatment. The suspensions obtained from the precipitates of unpooled acid gastric juice, however, were centrifuged and the clear supernatant solution only was given.

Assay of inhibitor activity was made according to a quantitative method developed by Code, Blackburn, Livermore and Ratke (7). For this procedure dogs with Heidenhain pouches were used, and the determination of inhibitor activity was made while these were secreting hydrochloric acid at fixed rates in response to repeated injections of histamine. In each assay the rate of gastric secretion employed was 41% or more of the maximal rate of output of the pouch. After a 2 hour control period during which secretion was maintained at this rate by injections of a fixed dose of histamine every 10 minutes, the material to be tested was given intravenously and the injections of histamine and collections of juice were continued for at least 3 more hours. Changes in output of hydrochloric acid during each of the subsequent 3 hours were expressed as per cent of the control rates, and the average of the changes in the second and third hours was used as a single value to express the inhibitor activity of the sample. As a rule the body temperature of the animals was determined by rectum 2 or more times during the control period and every half to one hour after the injection of the precipitate. The temperatures recorded during the control period were averaged and this value used for comparison with subsequent readings.

Results. Achlorhydric gastric juice from patients with pernicious anemia. Gastric juice obtained from 3 patients who had pernicious anemia was pooled in batch A and juice from 2 other patients was pooled in batch B. In the determinations of inhibitor activity carried out on dogs doses of 10 to 25 mg of the alcoholic precipitate obtained from these batches produced 60 to 86% inhibition of gastric secretion (Table I).

Achlorhydric gastric juice from persons without pernicious anemia or demonstrable disease of the gastro-intestinal tract. Three batches of precipitate were tested. The first, batch A, was prepared from juice which had been collected from 3 individuals and then pooled; the second, batch B, was obtained from 3 persons and the last, batch C, from 1 subject. Seven determinations of secretory depressant action were made (Table II). A large dose of 200 mg produced achlorhydria in the dog. Six tests with doses of 25 mg resulted in inhibition of secretion ranging from 55 to 75%.

Acid gastric juice from normal individuals. Precipitates were prepared from separate samples of the gastric juice of 4 persons and from 2 batches of pooled juice. The first,

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7. Code, C. F., Blackburn, C. M., Livermore, G. R., Jr., and Ratke, H. V., *Gastroenterology*, 1949, v13, 573.

TABLE II.
Gastric Secretory Inhibition Produced by Alcoholic Precipitates of Achlorhydric Gastric Juice from Otherwise Normal Human Beings.

Precipitate tested*			Assay		
Batch	Dose, mg	Dog	Control rate of secretion, % of max.	% inhibition	Max. rise of R.T.† °F
A	200	A	45	100	3.5§
A	25	E	‡	71	1.8
B	25	D	55	66	2.5
B	25	A	49	75	.4
B	25	C	58	74	.1
C	25	D	52	55	.6
C	25	A	50	75	.2

* For explanation see text.
† R.T.—Rectal temperature.
‡ Maximal rate of secretion not determined.
§ 4 episodes of emesis occurred.

TABLE III.
Gastric Secretory Inhibition Produced by 100 mg Doses of Alcoholic Precipitates of Acid Gastric Juice from Normal Subjects.

Precipitate tested*			Assay		
		Dog	Control rate of secretion, % of max.	% inhibition	Max. rise of R.T.† °F
Batch	A	E	‡	0	1.5
”	B	A	50	6	1.1
”	B	D	49	9	0.9
Subject	1	H	66	43	2.2§
”	2	L	51	0	0.7
”	3	V	47	41	2.7
”	4	L	54	16	1.2

* For explanation see text.
† R.T.—Rectal temperature.
‡ Maximal rate of secretion not determined.
§ Animal vomited twice.

batch A, was composed of juice collected from 2 individuals and the second, batch B, of juice secreted by 3 individuals (Table III). All of the juice contained acid in amounts customarily found in the gastric juice of normal individuals. The precipitates were given in doses of 100 mg. Significant amounts of inhibition were noted on 2 of the 7 tests (Table III).

Reactions. Only 2 immediate reactions occurred in the 18 assays. In the first, 200 mg of precipitate was given. This was the largest dose used in the entire series. Immediately after the injection the pulse and respiratory rates increased, the animal showed signs of weakness without lying down, salivated and defecated. About 30 minutes later the animal was alert and interested in its surroundings and within an hour had apparently fully recovered. Later the animal vomited several times. The other immediate reaction consisted of a brief period of pallor without apparent weakness or other symptom. A delayed rise in body temperature occurred in the course of all tests. In the course of half of the 10 tests in which the dose of the precipitates was 25 mg or less, the rise in body temperature was less than 1°F. The greatest increase was observed during the assay in which the largest dose of precipitate was used (Table II). When the maximal rise in body temperature observed on each test was plotted against the per cent inhibition of gastric secretion, no correlation between the 2 was apparent. (Values for each are given in Tables I, II and III.)

Comment. In general, the results of this investigation confirm and extend the findings of Brunschwig and his co-workers. The pronounced secretory inhibition produced by 10 mg doses of precipitates of achlorhydric gastric juice from patients with pernicious anemia is in agreement with their earlier observations. We have not tested achlorhydric gastric juice from patients with cancer of the stomach which was the other juice in which they found a high degree of inhibitor activity. We did find, however, that precipitates prepared from achlorhydric gastric juice of a group of persons whose hemopoietic and gastro-intestinal systems were normal produced pronounced inhibition while precipitates of acid juice of normal persons were much less active. The implications that high degrees of depressant activity may be expected in achlorhydric juice and that concentration of inhibitor and acid may be inversely related, however, are not fully supported by the findings of Kirsner, Nutter and Palmer (8). Their study led them to the conclusion that there was no direct correlation between pH and presence or absence of inhibitor activity in gastric juice of human beings. All of the patients of Kirsner, Nutter and Palmer who had pernicious anemia, however, had achlorhydria and all of the samples of gastric juice tested from these patients showed inhibitor activity. All samples of achlorhydric gastric juice from patients with pernicious anemia which have been studied by Brunschwig and his co-workers and by us have, likewise, shown inhibitor activity. One definite conclusion, then, seems warranted. Achlorhydric gastric juice of patients with pernicious anemia contains a substance or substances which are precipitated by alcohol and which when injected intravenously into dogs produce inhibition of gastric secretion.

The dogs used for tests of inhibitor activity by Brunschwig and his collaborators were secreting gastric juice in response to repeated feedings of small amounts of meat. With this stimulus inhibition of gastric secretion might conceivably be due to some inhibition of motility or digestion. The pronounced inhibition of histamine-provoked secretion produced by the injection of even small doses of active precipitates in our assays suggests rather more definitely than with secretion evoked by feeding that the precipitates have some direct action on the secretory mechanism in the gastric mucosa.

Summary. By use of a quantitative method for the determination of gastric secretory inhibitors the observation of Brunschwig and co-workers that the intravenous injection of alcoholic precipitates of achlorhydric gastric juice from patients with pernicious anemia depresses gastric secretion in dogs has been confirmed. The secretory depressant activity in such juice was shown by Brunschwig and his collaborators to reduce the secretion induced by feeding a meal. In this study it was found to be effective in inhibiting secretion provoked by histamine. The gastric secretory inhibitor was found in high concentration in achlorhydric gastric juice of persons without other demonstrable abnormalities in the gastro-intestinal tract or disease of the hemopoietic system. The gastric secretory inhibitor was absent or present in lower concentrations in gastric juice from normal individuals.

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Amino Acids of Bence-Jones Protein. (17865)

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The urinary excretion of Bence-Jones protein, in association with multiple myelomatosis, has been observed repeatedly(1,2), but few chemical investigations, in which present-day methods for the determination of its constituent amino acids were employed, have been reported. Recently Dent and Rose(3) have shown that the sample of Bence-Jones protein isolated by them contained no methionine. The absence of methionine (paper chromatography) was also reported by Ågren (4) and Papastamatis and coworkers(5). Harvier and Rangier(6) found 0.414% of methionine sulfur (equivalent to 1.80% methionine) but made no statement as to the method by which methionine was determined. In the sample of Bence-Jones protein isolated by Devine(7), 0.58% of methionine was present.

Since it is known that the proteins isolated from the urines in multiple myelomatosis differ in physico-chemical properties(2,7,8) and in immunological behavior(9,10), it seemed important to determine whether the absence (or very low content) of methionine is characteristic of all samples of Bence-Jones protein. Calvery and Freyberg(11), in the laboratory of one of us (L), studied the distribution of a limited number of amino acids in a sample of Bence-Jones protein. It was of interest to reexamine this protein by the newer methods of analysis (microbiological assay and two-dimensional paper chromatography). We have been able to account for about 92% of the amino acid residues of the protein. So far as it is known to us, this is the most complete analysis of the Bence-Jones protein which has been reported.

Experimental. Source of protein. The Bence-Jones protein was isolated from the urine of a patient with demonstrated multiple myelomatosis as previously described(11).

Hydrolysis of protein. Samples of the protein were hydrolyzed for 16 hr at 105° with 6N HCl in a sealed tube. The HCl was removed *in vacuo* and the residues were redissolved in water and evaporated repeatedly *in vacuo* to remove all free acid. Samples prepared in this manner were used for chromatography and for the microbiological determination of all of the amino acids except tryptophan.

For the determination of tryptophan, 22.8 mg of protein were placed in a pyrex test tube and dissolved in 0.5 ml of 5N NaOH by heating in a water bath. The mixture was autoclaved at 15 lb pressure for 16 hr, during which time complete racemization of tryptophan was assumed to have taken place. The digest was diluted, adjusted to pH 6.8, made up to 25 ml, and filtered through a dry filter

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TABLE I.
Description of Microbiological Assays.

Amino acid	Test organisms*	Standard amino acid†	Active conc. of reference standard,‡ γ/ml
Alanine	1	DL, Merck, recrystallized	30
Arginine	4	L, HCl	20
Aspartic acid	2	L	40
Cystine	1	L	5
	2	L	5
Glutamic acid	3	L	50
Glycine	2	Merck	10
Histidine	2	L, HCl, H_2O	5
Isoleucine	2	DL	15
Leucine	3	L	15
Lysine	2	L, HCl	50
Methionine	1	DL	8
	5	DL	8
Phenylalanine	2	DL	8
Proline	2	L	10
Serine	2	DL, Merck	20
Threonine	5	DL	15
Tryptophan	3	DL	3
Tyrosine	2	L	10
Valine	3	DL	15

* The test organisms in column 2 are listed as follows: 1, *Leuconostoc citrovorum* 8081; 2, *Leuconostoc mesenteroides* P-60; 3, *Lactobacillus arabinosus* 17-5; 4, *Lactobacillus delbrueckii* 3; 5, *Streptococcus faecalis* R.

† Unless otherwise stated, the amino acids were present in a specially tested composite standard obtained from Dr. L. M. Henderson.

‡ The highest concentration of any amino acid per assay tube was one-half that of the reference standard. It was assumed that the D- and L-isomers of alanine had equal activity for the organism used. For all other DL-isomers, the D-form was considered inactive (16,17).

to remove the silica. The filtrate was diluted suitably for microbiological assay. The value obtained was doubled to give the original L-tryptophan content of the protein.

Chromatography. Volumes of the acid hydrolysate corresponding to 1 mg of the original protein (148 γ of N) were placed on paper for 2-dimensional paper chromatography by the method of Consden, Gordon, and Martin(12). Phenol saturated with water was used as the first solvent and a mixture of collidine and lutidine saturated with water as the second. The spots were treated with hydrogen peroxide(13) prior to chromatography to convert methionine to the sulfone and cystine to cysteic acid. Several samples were treated with hydrogen peroxide and molybdate to insure complete conversion of all of the methionine to the sulfone(14).

Microbiological procedures. The data pertinent to the determination of the individual amino acids are summarized in Table I. The micromethods of Henderson, Brickson, and Snell(15) and Steele, Sauberlich, Reynolds, and Baumann(16) were employed throughout with the basal medium recommended by Henderson and Snell(17). When *L. citrovorum* was used as the test organism(16), the medium was supplemented with a commercial liver preparation (Reticulogen). Alanine was assayed by the method of Sauberlich and Baumann(18) with the use of the

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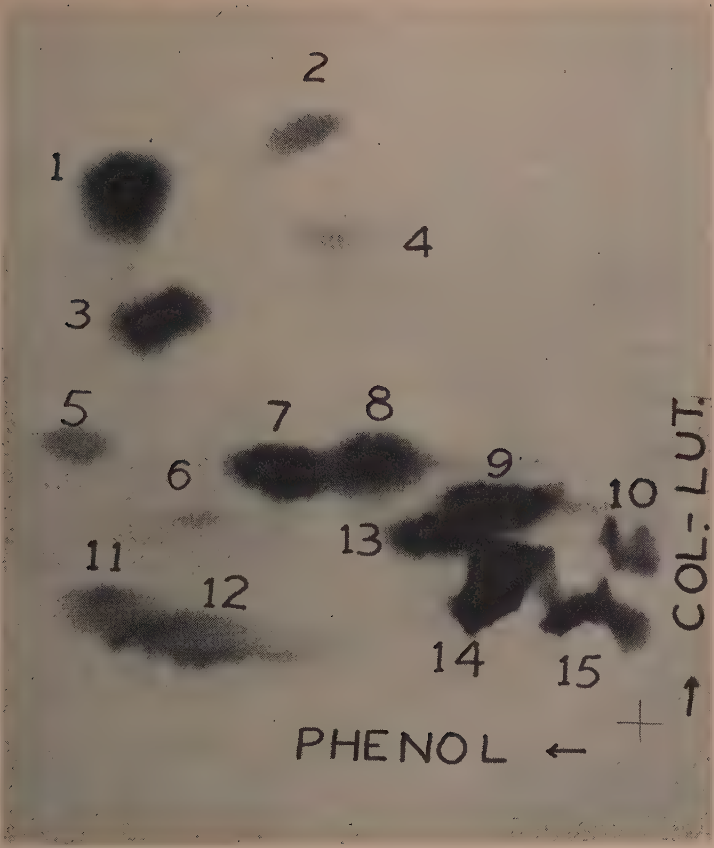

FIG. 1.
Chromatogram of peroxide-treated hydrolysate of Bence-Jones protein. Leucine and isoleucine, 1; tyrosine, 2; valine, 3; glucosamine, 4; proline, 5; histidine, 6; alanine, 7; threonine, 8; serine, 9; cystine (cysteic acid), 10; arginine, 11; lysine, 12; glycine, 13; glutamic acid, 14; aspartic acid, 15.

same organism. Although the blanks were somewhat high, the assays were entirely satisfactory. In the calculation of the results it was assumed that the D- and L-alanine have equal activity for *L. citrovorum* and that all other D- amino acids are completely inactive. Each value reported represents the mean of determinations made at 2 dilutions. The values for each dilution are based on the titration of 18 tubes, *i.e.*, a minimum of 36 determinations for each amino acid was actually carried out. In numerous instances the assay was repeated 2 or 3 times. In view of the importance of the methionine determination, the assays for this amino acid were performed with 2 different microorganisms. The relatively good checks obtained with *L. citrovorum* and *S. faecalis* R make it seem improbable that the active material was an artifact. Good checks were also obtained when cystine was determined with *L. citrovorum* and *L. mesenteroides*.

Other analyses. The water content of the protein was measured by drying to constant weight at 60° *in vacuo*. The ash was estimated by ignition to constant weight at 450° in a muffle furnace. The nitrogen content was determined by a micro-Kjeldahl pro-

TABLE II.
Amino Acid Composition of Bence-Jones Protein.
The ash content was 3.34%; moisture, 7.89%; nitrogen corrected for ash and moisture, 16.70%. All values in Table II are expressed on an ash-free and moisture-free basis.

Amino acid	G per 100 g protein		G residue per 100 g protein	G N per 100 g protein
Glycine	5.41		4.11	0.77
Alanine	8.23		6.56	1.29
Valine	6.87		5.81	0.70
Leucine	6.82		5.88	0.63
Isoleucine	3.61		3.12	0.39
Phenylalanine	2.93		2.61	0.25
Proline	7.10		5.98	0.73
Tryptophan	2.25		2.04	0.31
Cystine	2.71	2.93* 2.48†	2.51	0.30
Methionine	0.12	0.11* 0.13‡	0.11	0.01
Glutamic acid	12.28		10.79	1.17
Aspartic acid	7.10		6.14	0.75
Histidine	1.91		1.69	0.52
Lysine	6.25		5.47	1.20
Arginine	5.07		4.54	1.63
Serine	12.61		10.48	1.69
Threonine	8.35		7.09	0.98
Tyrosine	5.41		4.88	0.42
Amide-NH_3	2.13		2.13	1.75
Total	107.16		91.94	15.49

* *L. citrovorum* 8081.
† *L. mesenteroides* P-60.
‡ *S. faecalis* R.

cedure. Amide nitrogen determinations were made on the acid hydrolysates by the Conway micro-diffusion method(19). Spectrophotometric examination of solutions of the acid hydrolysates failed to reveal any significant absorption in the ultraviolet attributable to purines and pyrimidines.

Chromatographic findings. Numerous chromatograms were made of acid hydrolysates of the Bence-Jones protein. A typical chromatogram is shown in Fig. 1. In confirmation of previous reports(3-5), methionine and hydroxyproline were not detected in any of the samples. Although phenylalanine is not visible on the photograph, it was present on the original paper as a light halo above the leucine spot. Phenylalanine appeared clearly as a separate spot in other chromatograms which were allowed to run in collidine for longer periods of time. Spot 4 has been tentatively identified as glucosamine. It was found that no new spots appeared when chromatograms were made of samples to which glucosamine had been added and that spot 4 was intensified. Dent also reported the presence of carbohydrate in his sample (3). The large quantities of serine, threonine and proline were of special interest. It is not possible to compare the intensity of the proline spot (spot 5) on the photograph with that of the other amino acids, since proline gives a yellow color while the other amino acids give colors ranging from blue to purple. Tryptophan was not detected since this acid was destroyed during acid hydrolysis.

A semi-quantitative estimation of the amounts of the various amino acids from the chromatograms was of considerable help in establishing the quantities of hydrolysate to be used in the microbiological assays.

Microbiological determinations. It was possible to account for 92% of the weight of the protein and 93% of the nitrogen from the analyses listed in Table II. The methionine assays using two different organisms were in

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19. Conway, E. J., Microdiffusion Analysis and Volumetric Error, Crosby Lockwood, & Son, Ltd., London, 1947.

excellent agreement. The amount of methionine found was below the limit of detection of the chromatographic method for the quantities of hydrolysate examined. There was complete qualitative agreement between the chromatographic results and the microbiological assay in the estimation of the relative amounts of the various amino acids.

Discussion. Some analyses have been published previously for the sample of Bence-Jones protein employed in the present study (11). The previous finding of unusually high total nitrogen (18%) and amide nitrogen (5.7%) values has not been confirmed. Significantly lower values of 16.7% and 2.13%, respectively, have been obtained in replicate analyses. The microbiologically determined values for tyrosine, tryptophan, cystine, arginine, histidine, and lysine are in fairly good agreement with those previously reported, while the aspartic and glutamic acid values are considerably higher. Tyrosine and tryptophan have been determined spectrophotometrically, by a procedure in which no hydrolysis of the protein is required, by Mr. D. M. Teague of the Physical Chemical Research Laboratory of the Chrysler Corporation and the laboratory of one of us (L.). Corrected values for the Bence-Jones protein are tyrosine, 6.34%, and tryptophan, 4.34% (unpublished data). Although, in the present study, no methionine was detected by paper chromatography, closely agreeing microbiological assays with two different organisms gave a mean value of 0.12%, an amount below the limit of detection by the chromatographic method. Our small, but definite, values may reflect a difference in the proteins from the two sources or may be the result of the presence of an impurity in our proteins. All of our findings are in essential agreement with those of Dent and Rose(3). No hydroxyproline was detected. Large quantities of serine and threonine were found. The microbiologically determined value for cystine was 2.71% as compared to 2.47%, obtained by chemical analysis in their studies. A comparison of the amino acid composition of our sample of Bence-Jones protein with that of other proteins shows more points of similarity with human γ-globulin than with any of the other proteins cited in Tristram's compilation(20). The high content of serine and threonine in both of these proteins is of special interest, since 3 enzymatically active proteins, pepsin, chymotrypsinogen, and ribonuclease are also reported to have unusually high contents of these amino acids(20).

The examination of the analytical values for the sample of Bence-Jones protein studied does not allow speculation about the etiology of multiple myelomatosis based solely on the similarities in amino acid composition to those of γ-globulin(11) or viruses(3). It may be pointed out, however, that the similarity between virus proteins and Bence-Jones protein(3) with relation to their methionine content may be extended by the present work to include a second common factor, a relatively high content of the aliphatic hydroxy amino acids(21). It may be noted that, with the exception of one strain, the tobacco mosaic virus proteins analyzed by Knight(21) contained a high content (over 16%) of aliphatic hydroxy amino acids. The high content of these acids in Bence-Jones protein is another point of similarity to the virus proteins, in addition to the absence or very low content of methionine(3).

Summary. 1. Hydrolysates of a sample of Bence-Jones protein isolated from the urine of a patient with multiple myelomatosis were examined by paper chromatography. Quantitative estimations of 18 amino acids were also made by microbiological methods.

2. The protein was characterized by a very low content of methionine (0.12%) and by relatively large amounts of serine, threonine, and proline. No evidence of the presence of hydroxyproline was obtained.

3. The analyses accounted for 93% of the nitrogen present in the protein.

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Changes in Cell and Plasma Volumes Produced by Total Body X Radiation. (17866)

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There have been many reports on alteration in cellular and chemical composition of the blood following total body irradiation. The interpretation of such data, notably those recording only slight changes, is not possible unless the total plasma and cell volumes are known. A systematic study of the blood volume following irradiation has thus far, to our knowledge, not been made, although reference is made to some plasma volume determinations in X-rayed animals by Muntz and associates(1).

Procedures. The red cell volume was determined by the introduction of ^{32}P-labeled homologous erythrocytes as described by Hevesy(2), and modified by Nieset *et al.* (3). The plasma volume was determined by the Evans blue (T-1824) technic and in some instances by a modification of the technic of Fine and Seligman(4) utilizing plasma tagged with ^{131}I. The optical density of Evans blue solution was determined in a Beckman spectrophotometer using a Lowry cell(5) as described elsewhere(6).

The hematocrits were made with the capillary technic of Parpart and Ballentine(7).

The factors of irradiation were as follows: *Mice*: 250 kv; 12½ ma; tsd 90 cm; 1.5 mm Cu; inherent filtration 3 mm Al. Output 22.3 r per minute. *Rabbits*: 250 kv; 15 ma; tsd 53.5 cm; nofiltration other than above inherent filtration. Rate 91 r per minute. After 500 r exposure the rabbit was turned over.

The plasma and cell volume determinations were made in the same animal concurrently. Preliminary experiments have indicated that it was immaterial whether one or the other material was injected first. All rabbits were females. They were injected in one ear vein and sampled from the opposite ear vein. All mice were males of the Rf stock. They were injected into the tail vein and sampled from either the jugular or the heart blood.

Results. Fig. 1 is a graphic record of the sequence of cell, plasma and total blood volume changes in female rabbits following total body irradiation with 1,000 r. The upper part of the figure gives values calculated on the basis of injected ^{32}P-tagged erythrocytes; the lower part gives values based on the conventional Evans blue technic. The ex-

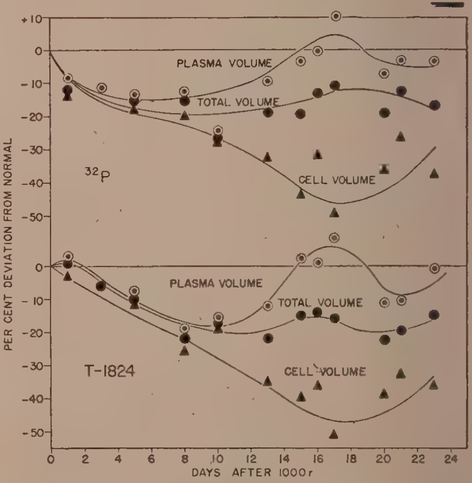

Fig. 1.

Cell, plasma, and total-blood volume of rabbits exposed to 1,000 r calculated on basis of preirradiation weights and expressed in percent deviation from normal.

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TABLE I.
Cell, Plasma, and Total-Blood Volumes in X-Rayed Rabbits.

		Volume—% body wt							
				Total			Body wt		
Days after irrad.	No. in group	Cell by ^{32}P	Plasma by T-1824	by ^{32}P	by T-1824	Combined (corrected)	Before irrad.	At test	Hematocrit
0	8	1.96	4.06	4.87	6.80	5.72	—	3430	40.2
1	2	1.67	4.18	4.28	6.84	5.54	3516	3308	39.1
3	2	1.74	3.81	4.32	6.40	5.27	3393	3140	40.6
5	2	1.61	3.74	4.14	6.15	5.07	3675	3437	39.3
8	2	1.57	3.29	4.13	5.31	4.59	3822	3572	38.2
10	2	1.41	3.43	3.59	5.65	4.59	3742	3459	39.6
13	2	1.32	3.57	3.95	5.34	4.62	3738	3484	33.3
15	2	1.11	4.15	3.91	5.81	4.95	3055	2774	28.4
16	2	1.35	4.09	4.24	5.85	5.13	3675	3596	32.3
17	2	1.00	4.40	4.36	5.74	5.07	3742	3325	21.4
20	2	1.25	3.61	3.95	5.29	4.59	3648	3162	31.3
21	2	1.45	3.64	4.27	5.50	4.83	3737	3750	33.9
23	2	1.23	4.04	4.05	5.79	4.97	3393	3024	30.3

The values are expressed as percent body weight immediately before irradiation. The combined corrected total-blood volume is the sum of the cell volume determined by ^{32}P-labeled erythrocytes and the plasma volume by T-1824 divided by 1.08. The latter is the correction factor for Evans blue withdrawn from blood 5-6 minutes after injection—as indicated by the presumably more correct ^{131}I-plasma technic(11).

perimental data are shown in Table I. The graphs are based on calculations using the body weight immediately before irradiation, since the animals lose weight during the first few days after irradiation apparently without losing blood. There is an apparent rise of blood volume if expressed in percent of body weight at time of determination.

Fig. 1 and Table I indicate a marked cell volume drop beginning soon after irradiation and reaching a depth at about the 17th day. The plasma volume drops more slowly and reaches a depth at about the 10th day, after which it rises. Concomitant with the latter there begins an elevation of the total-blood volume which has not reached the normal in 23 days.

It remains to determine how much of the red cell volume drop is due to a dropping out of aged cells while the marrow is depleted and how much to red cell injury. Erythrocytes are believed to be relatively resistant to X-rays but it is possible that irradiation hastens their death. The plasma volume drop

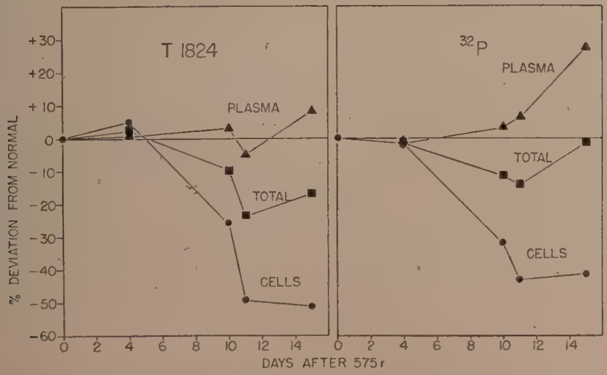

Fig. 2.

Cell, plasma, and total-blood volume of mice exposed to 575 r calculated on basis of preirradiation weights and expressed in percent deviation from normal.

TABLE II.
Cell, Plasma, and Total-Blood Volumes in X-Rayed Mice.

		Volume—% body wt						
				Total				
Days after irrad.	No. in group	Cell by ^{32}P	Plasma by T-1824	by ^{32}P	by T-1824	Combined (corrected)	Body wt at test	Hematocrit
0	7	2.67	5.47	6.39	9.46	7.22	29.9	42.0
4	6	2.62	5.51	6.29	9.70	7.22	26.6	41.9
10	2	1.82	5.64	5.66	8.56	6.52	27.9	31.2
11	3	1.52	5.20	5.48	7.23	5.86	27.6	27.9
15	3	1.56	5.94	6.31	7.89	6.51	24.8	24.6

The values are expressed as percent body weight immediately before irradiation. The combined total-blood volume is the sum of the cell volume determined by ^{32}P-labeled erythrocytes and the plasma volume by T-1824 divided by 1.2. The latter is the correction factor for Evans blue withdrawn from the blood 5-6 minutes after injection as indicated by the presumably more correct ^{131}I-technic.

and the subsequent rise likewise require further analysis. Changes similar to those found in rabbits were observed in mice which received 575 r over the entire body. (Fig. 2 and Table II). Reticulocytes are known to be depleted after irradiation during 1-2 weeks (8,9,10). Thus the drop during about the 2-8-day period may be a measure of the sum of the erythrocytes dying because of aging and of irradiation damage.

Observations on the rate of disappearance of material used for blood volume determinations. In preliminary experiments the rate of disappearance of the substances used for blood volume determinations was assayed in both mice and rabbits at various intervals after irradiation. The blood-volume values are based on samplings at 5 to 6 minutes with all materials used. Such samplings made with tagged erythrocytes and plasma probably give more correct values while the Evans blue drop from 1- to 2-minute peak is due to removal of the dye from the circulation by Evans blue-affin cells. We believe that the peak Evans blue value following its introduction would more closely express the correct plasma volume but, since this point is difficult to determine, the conventional Evans blue technic was adhered to. Data to be described indicate that the Evans blue value divided by 1.2 in mice and 1.08 in rabbits equals the ^{131}I plasma value(11).

The 30-minute rate of disappearance of the injected material was assayed in a large number of animals. The results were highly variable but the sum of the data suggests that often there is a greater disappearance of these materials in the irradiated than in the normal mice and rabbits. These data will be fully reported in the final paper, and will be correlated with drop of red cell counts following irradiation and calculations on the aging rate of the erythrocytes and the influence of irradiation on their survival time.

Summary and conclusion. Following irradiation of rabbits by 1,000 r of X-rays there is a large drop in erythrocyte volume as measured by ^{32}P-tagged cells beginning with the 3rd day and reaching a depth at about the 17th day after irradiation, followed by a slow recovery. There is at first a slight concomitant plasma-volume drop followed by a rise on about the 10th day that elevates the lowered total volume but does not bring it up to normal in 23 days.

Similar changes occur in mice that have been exposed to 575 r.

Mary Knoohuizen and Maryann Huddleston assisted in the work.

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Effect of ACTH on Wound Healing in Humans.* (17867)

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In an earlier report(1), the chance observation of delayed healing of wounds in patients on adrenocorticotrophic hormone (ACTH) therapy was noted. These wounds had occurred as a result of complications unrelated to the basic disease being treated with ACTH. In a later report(2), controlled studies in rabbits were recorded and again delay in connective tissue formation in cortisone treated animals was noted. It is the purpose of this report to present the results of controlled studies in human subjects.

In 2 patients treated with ACTH* (one with periarteritis nodosa and one with rheumatoid arthritis) elliptical skin biopsies were made on the first day of therapy. The

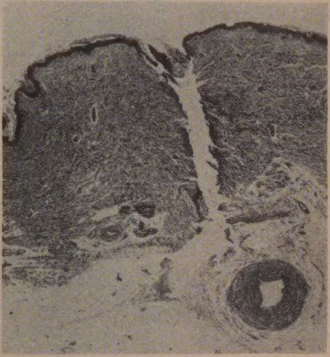

Fig. 1.
One-week-old skin wound from patient on ACTH: Hematoxylin and eosin, × 11.

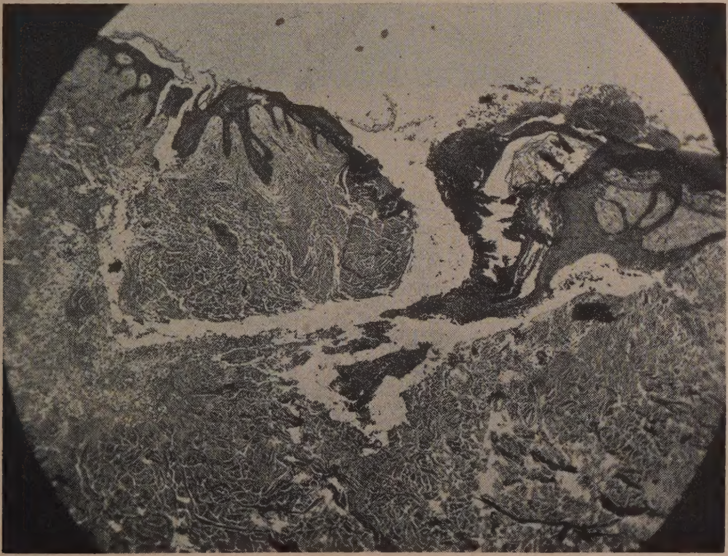

Fig. 2.
One-week-old skin wound from patient on ACTH. Hematoxylin and eosin, × 38.

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* Aided in part by a grant from the Masonic Foundation for Medical Research and Human Welfare.

1. Ragan, C., Grokoest, A. W., and Boots, R. H., *Am. J. Med.*, 1949, v7, 741.

2. Ragan, C., Howes, E. L., Plotz, C. M., Meyer, K., and Blunt, J. W., Proc. Soc. Exp. Biol. and Med., 1949, v72, 718.

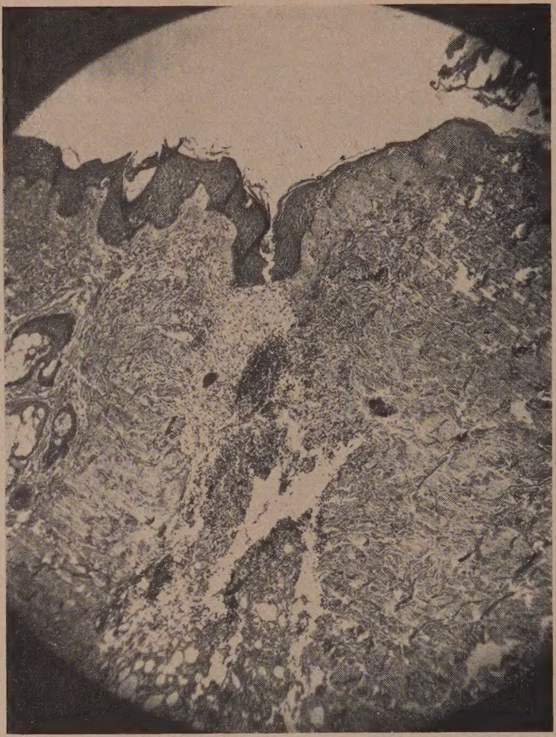

FIG. 3.
One-week-old incised wound 1 week after cessation of ACTH. Hematoxylin and eosin, × 38.

wounds were closed with silk and after one week the whole area was excised. The dose of ACTH during this time was 100 mg per day on the first day and 40 mg per day thereafter. Under normal circumstances such wounds should be either healed or show evidences of advanced granulation tissue formation after one week.

Fig. 1 and 2 are photo-micrographs of the re-biopsied wounds. As can be seen in cross section, there is no evidence of healing of the mesenchymal tissues. A small amount of fibrin is seen between the wound edges. There is proliferation of epithelial structures, but practically no granulation tissue, leucocytes, fibroblasts or proliferating blood vessels are evident. One week later and one week after cessation of ACTH, the wound sites were re-examined. The first patient's wound was grossly healed and in the second a re-biopsy was performed as a further control. Histologically, the normal healing was evident (Fig. 3).

In an attempt to reverse this action of ACTH an additional experiment was performed. Seifter *et al.*(3,4) have implied, as a result of *in vitro* and *in vivo* studies, that cortisone and certain other steroids cause inhibition of hyaluronidase activity. It was therefore postulated that hyaluronidase activity might be necessary for proper wound healing and that inhibition of such activity might have been responsible for the observed effects of ACTH. Therefore, in a 45-year-old female patient with rheumatoid arthritis on ACTH, an incised wound 2 cm long and about 1.5 cm deep was made on the lateral aspect of the right thigh. An ethylene plastic catheter corresponding in size to a 20-gauge needle was inserted obliquely into the wound and the wound edges were then closed with silk around the catheter. Using aseptic precautions the wound was irrigated through the catheter 6 times daily with 0.5 cc of a solution containing 90 turbidity reducing units of bull testes hyaluronidase† and 50,000 units of penicillin.

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* The ACTH used in this study was generously supplied by Dr. John R. Mote of the Armour Laboratories.

3. Seifter, J., Baeder, D. H., and Dervines, A., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 136.

4. Seifter, J., Baeder, D. H., and Begany, J., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 277.

In this rather gross manner an attempt was made to supply an excess of exogenous hyaluronidase to the tissue which might be theoretically depleted in endogenous enzyme. On the opposite leg a control incision was made and irrigated with penicillin solution alone. After 4 days both wounds were excised. The control wound appeared much the same as those described above. The hyaluronidase-treated wound appeared to be healed grossly. After removal of the sutures the wound edges adhered. Fig. 4 is a photomicrograph of a cross section through the wound. The wound edge is bridged by a continuous layer of epithelium. However, the subepithelial layers are filled only with fibrin with no evidence of fibroblastic activity. Incised wounds of rabbits treated with cortisone have also failed to heal when hyaluronidase was injected locally into the wound.

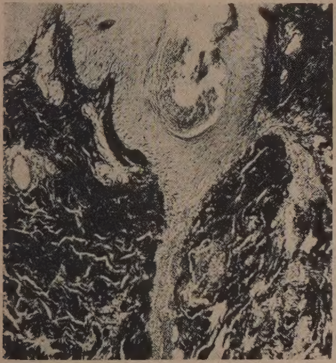

Fig. 4.
4-day-old wound locally treated with hyaluronidase (see text). Trichrome stain, × 68.

The significance of this "false healing" is unknown and may have been merely fortuitous. However, it does illustrate that, in spite of epithelialization and superficial evidence of gross healing, "true healing" in terms of fibroblastic activity failed to occur.

In all of the patients studied the usual clinical and laboratory evidences of hyperadrenalism occurred. Dramatic improvement in the disease processes took place. The circulating eosinophile counts dropped significantly. The erythrocyte sedimentation rates fell and the characteristic electrolyte and uric acid excretory changes were seen.

Discussion. These observations indicate that wound biopsies provide a readily available means for study of the actions of ACTH upon tissues. Moreover, wound healing may be considered a type of tissue reaction somewhat analogous to that abnormally occurring in the mesenchymal diseases susceptible to therapy with ACTH. As such, it constitutes a tool for further study of the mechanism of the therapeutic effects of ACTH.

Summary. Observations are presented indicating that during hyperadrenalism induced by ACTH there is inhibition of wound healing in humans. The value of these observations as a means of studying the effects of hyperadrenalism at the tissue level is implied.

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† Supplied as "Hydase" by Wyeth Co.

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